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Down regulation of gene expression in *Toxoplasma gondii* by engineered delta ribozymes.

Ju. Sheng
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**DOWN REGULATION OF GENE EXPRESSION IN
TOXOPLASMA GONDII BY ENGINEERED
DELTA RIBOZYMES**

by Ju Sheng

A Thesis Submitted to the Faculty of Graduate Studies and Research through the
Department of Chemistry and Biochemistry in Partial Fulfillment of the Requirements for
the Degree of Master of Science at the
University of Windsor

Windsor, Ontario, Canada

2003

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ABSTRACT

DOWN REGULATION OF GENE EXPRESSION IN *TOXOPLASMA GONDII* BY ENGINEERED DELTA RIBOZYMES

JU SHENG

Delta ribozyme is a RNA enzyme derived from the genome of hepatitis delta virus (HDV). It can specifically cleave a RNA substrate and is the only ribozyme that can function naturally in human cells. These properties make the delta ribozyme attractive as a gene modulator. *Toxoplasma gondii*, a pathogenic parasite to humans and pets, has been used for genetic studies. Genes encoding uracil phosphoribosyltransferase (UPRT) and hypoxanthine-xanthine-guanine phosphoribosyltransferase (HXGPRT), which are the operative enzymes of the pyrimidine and purine salvage pathway, respectively, in *T. gondii*, were chosen for the study of the down regulation effect of delta ribozymes. Different delta ribozymes were engineered to recognize different locations on the *UPRT* and *HXGPRT* mRNA. Cleavage assays of the ribozymes using RNA substrates (ranging from 15 nt to 725 nt) have been carried out. One engineered ribozymes exhibited the highest cleavage activity. Primer extension reactions indicated that the cleavage occurred at the expected site.

The engineered ribozymes were electroporated into the parasites. UPRT activity was monitored by measuring ^3H -uracil incorporation, while HXGPRT activity was monitored by measuring ^3H -hypoxanthine incorporation. UPRT and HXGPRT activities were reduced in parasites electroporated with engineered delta ribozymes as compared to controls. Furthermore, UPRT and HXGPRT activities were inhibited simultaneously when two engineered delta ribozymes, which against *UPRT* and *HXGPRT* targets

respectively, were electroporated into parasites. Northern blots confirmed that the reduction in UPRT and HXGPRT activities were due to lower levels of *UPRT* and *HXGPRT* mRNA.

In addition, a stable cell line of *T. gondii* expressing the engineered delta ribozymes against *UPRT* was established. The plasmids carrying engineered delta ribozymes was maintained in the transformed parasites. The expression of delta ribozymes in these parasites could be detected by RT-PCR. In conclusion, the engineered delta ribozymes displayed cleavage activity *in vitro* and down regulation effect on the gene expression in *T. gondii*. It is thus suggested that delta ribozyme could be potentially used as a therapeutic tool to target genes that are essential for the parasite growth and survival.

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LIST OF ABBREVIATIONS

6TX	6-thioxanthine
AAV	adeno-associated virus
ATP	Adenosine triphosphate
CAT	chloramphenicol acetyl transferase
cDNA	complementary DNA
CIAP	calf intestinal alkaline phosphatase
CPM	count per minute
CSPD	1,2-dioxetane chemiluminescent enzyme substrate
DEPC	diethylpyrocarbonate
DHFR-TS	dihydrofolate reductase-thymidylate synthase
DIG	digoxigenin
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxyribonucleoside triphosphate
DPBS	Dulbecco's phosphate-buffered saline
<i>E.coli</i>	<i>Escherichia coli</i>
EDTA	ethylenediaminetetra-acetic acid disodium salt
FBS	fetal bovine serum
dUMP	deoxy uridine monophosphate
FDUR	fluorideoxyuridine
HBV	hepatitis B virus
HDV	hepatitis delta virus
HFF	human foreskin fibroblasts
HXGPRT	hypoxanthine-guanine phosphoribosyltransferase
Min	minutes
M-MLV RT	Moloney murine leukemia virus reverse transcriptase
Mmol	millimole
MOPS	3-(N-morpholino) propanesulfonic acid
MPA	mycophenolic acid
mRNA	messenger RNA
nt	nucleotide
NTP	ribonucleoside triphosphate
Oligo	oligonucleotide
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PRPP	α -D-5-phosphoribosyl-1-pyrophosphate
RH	wild type
RNA	ribonucleic acid
RNase	ribonuclease

ROP	roptry protein
RT	reverse transcription
RT-PCR	reverse transcription PCR
SAG	surface antigen
Sec	second
SSC	sodium chloride-sodium citrate
TAE	Tris-Acetate-EDTA
<i>Taq</i>	<i>Thermus Aquaticus</i>
TBE	Tris Borate EDTA
TCA	trichloroacetic acid
TEMED	N,N,N',N'-Tetramethylethylenediamine
TMP	Thymidine monophosphate
tRNA	transfer RNA
TUB	tubulin
UMP	Uridine monophosphate
UPRT	uracil phosphoribosyltransferase
UV	ultraviolet

CHAPTER 1

INTRODUCTION

Toxoplasma gondii is a worldwide pathogenic parasite of humans and some pets (Luft and Remington, 1992). Normally, this parasite is controlled by the immune system of healthy individuals. Antibiotics treatment is recommended. However the treatment is not always effective, especially for the recurrent infection. Here a new molecular technique has been applied in the investigation of gene function and down-regulating the expression of the genes required for the parasite invasion and growth.

Ribozymes have been widely recognized as potential therapeutical tools in the treatment of human disease. Delta ribozymes are a unique class of ribozymes because they are the only catalytic RNAs known to be naturally active in human cells (Sun et al., 2000).

In this study we investigate the down-regulation effect of delta ribozymes on the gene expression in *T. gondii*. Genes encoding uracil phosphoribosyltransferase (UPRT) and hypoxanthine-xanthine-guanine phosphoribosyltransferase (HXGPRT), which are the operative enzymes of the pyrimidine and purine salvage pathway, respectively, are chosen for the study. This chapter will review the materials pertaining to this study and will outline the objectives of the study.

1.1 *Toxoplasma gondii*

Toxoplasma gondii, a member of the phylum Apicomplexa, is a globally widespread intracellular protozoan parasite (Luft and Remington, 1992). It causes significant disease in many warm-blooded animals and humans. Infections are commonly acquired by ingestion of under-cooked meat or by contact with cats which carry parasite oocysts. It is estimated that one-third of North-Americans and up to

70% of certain European countries have been infected by this parasite (Remington et al., 1995). Infections in healthy adults are usually asymptomatic. Pregnant women infected with *T. gondii*, however, may risk the congenital toxoplasmosis which results in the severe injury of the foetus. Especially it is a leading opportunistic infection associated with AIDS (Luft and Remington, 1992), and cancer patients (Israelski and Remington, 1993).

1.1.1 Life cycle of *Toxoplasma gondii*

T. gondii is a unicellular eukaryotic parasite. It is an obligate intracellular existence throughout its life cycle. *T. gondii*'s life cycle is rather complex involving two independent cycles (asexual and sexual). The sexual cycle involves gametogenesis, zygote formation and development into an oocyst that exists in the intestinal epithelial cells of felines. The asexual cycle can possibly occur in almost any warm-blooded animal. During infection in the intermediate host, it undergoes stage conversion between the rapidly dividing tachyzoite (responsible for acute illness) and the slowly replicating encysted bradyzoite stage. It is likely that tachyzoites change into bradyzoites is influenced by the host's immune response and chemotherapy. The tachyzoite-bradyzoite interconversion process involves in several stage-specific genes and proteins (Tomavo, 2001; Roos et al., 1994).

1.1.2 *T. gondii* as an experimental model

Many pathogenic protozoans of human and animals (particularly intracellular parasites) are difficult to culture in laboratory. The studies of mechanisms of pathogenesis and control for these organisms are thus extremely difficult (Sibley et al., 1996). *T. gondii*, however, is readily cultured in human foreskin fibroblasts (HFF) or

other nucleated cells (Boothroyd et al., 1994). The tachyzoite form is capable of rapid asexual replication in 3-5 days, qualifying *T. gondii* as an inexpensive experimental organism.

T. gondii is readily mutagenized which makes it ideal for genetic studies (Pfefferkorn, 1983). The genome of *T. gondii* has been well documented. In tachyzoite form, *T. gondii* has a haploid genome, consisting of approximately 80 Mb in 11 chromosomes (Ajioka et al., 1998).

1.1.3 Genetic manipulations of *T. gondii*

The molecular transformation of *T. gondii* has proved to be successful and feasible in several studies (Soldati and Boothroyd, 1993; Donald and Roos, 1993; 1994; Kim et al., 1993). A series of transformation vectors have been developed for *T. gondii*, for example, the pyrimethamine resistance vectors pDHFR-TS based on Bluescript pSK plasmid (Donald and Roos, 1993). Electroporation is an efficient way to introduce the plasmids into parasites. The recombinant plasmid could be expressed either transiently or permanently leading to the production of stable transformants. Plasmids can be maintained as episomes or integrated into the parasite genome as single copy or multicopy transgenes (Donald and Roos, 1993).

1.1.3.1 Transient expression

Plasmids, carrying a bacterial chloramphenicol acetyl transferase (CAT) reporter gene under control the promoter of P30 major surface antigen (SAG1), have been successfully introduced into tachyzoites by electroporation and can be transiently expressed. The promoters normally used are SAG1, dihydrofolate reductase-

thymidylate synthase (DHFR-TS), a rhoptry protein (ROP1), and β -tubulin (TUB1) (Roos et al., 1994; Soldati et al., 1998).

1.1.3.2 Stable transgene expression and markers for selection

Stable transformants can be selected by maintaining transfected parasites in a selection pressure (i.e chemicals such as chloreamphenicol for CAT). After introducing the plasmids into *T. gondii* tachyzoites by electroporation, the purified clonal progeny of individual transformants can be obtained by lysate plaque screening several times in the presence of a selection drug (Pfefferkorn et al., 1978). The transformants remain drug-resistant even in the absence of continued selection (Donald and Roos, 1993, 1994). Many reports demonstrated the feasibility of obtaining stable transformants (Donald and Roos, 1993; Kim et al., 1993; Sibley et al., 1994; Messina et al., 1995; Soldati et al., 1995). Nonessential nucleotide salvage pathway enzymes, such as UPRT and HXGPRT, are often used as positive or negative selectable markers for *T. gondii* transformation (Donald and Roos, 1994). Mutants defective in UPRT can be obtained by 5-fluorodeoxyuridine (FUDR) selection (Donald and Roos, 1995). Mutants of HXGPRT can be selected by addition of either 6-thioxanthine (6-TX) or mycophenolic acid (MPA) to the culture (Donald et al., 1996). Other markers, such as the negative selectable marker thymidine kinase, were also developed for selecting stable DNA transformation of *T. gondii* (Roos et al., 1994; Radke et al., 1998).

1.1.3.2.1 Uracil Phosphoribosyltransferase (UPRT)

UPRT is a 244-aa protein (molecular mass, 27 kDa; Cater et al., 1997) that belongs to the nucleobase phosphoribosyltransferase family. Normally it catalyzes the

conversion of uracil to UMP during *T. gondii*'s pyrimidine salvage pathway (Roos et al., 1994). UPRT is the only operative enzyme of the pyrimidine salvage pathway that salvages performed pyrimidines at the nucleotide level. The reaction it catalyzes involves phosphoribosyl transfer from the substrate α -D-5-phosphoribosyl-1-pyrophosphate (PRPP) to an acceptor molecule (Iltzsh et al., 1993).

UPRT can recognize variety analogs of pyrimidine nucleobase. In *T. gondii*, UPRT catalyzes the phosphoribosylation of 5'-fluorouracil, an analogue of uracil, to fluorouridine leads to the synthesis of F-dUMP and subsequent fatal inhibition of its TMP synthesis (Iltzsh et al., 1994). As *Toxoplasma* can synthesize pyrimidines *de novo* (Schwartzman et al., 1981), UPRT is not essential for the parasite. Mutants deficient in UPRT activity have been isolated by both chemical mutagenesis and molecular genetic strategies (Pfefferkorn et al., 1977; Schwartzman et al., 1981; Donald and Roos, 1995). These mutants are resistant to fluorideoxyuridine (FDUR) and unable to incorporate radiolabeled uracil.

Mammalian cells, instead of using UPRT, can convert uracil to UMP through the action of uridine phosphorylase and uridine kinase. Since both uridine phosphorylase and uridine kinase can not recognize 5'-fluorouracil, there is no toxic product produced in mammalian cells (Pfefferkorn et al., 1983). Therefore, UPRT can be used as a negative selectable marker. Most important, UPRT in *T. gondii*, which is absent in humans, offers a promising target for the study of down-regulation effect of genetic tools.

1.1.3.2.2 Hypoxanthine-Xanthine-Guanine Phosphoribosyltransferase (HXGPRT)

T. gondii has phosphoribosyltransferase activity for hypoxanthine, xanthine, and guanine presumably within the same protein (Donald et al., 1996). Gene that encoding

HXGPRT was studied by insertional mutagenesis showing that mutants always lack activity to incorporate hypoxanthine, xanthine and guanine for all three nucleotides (Pfefferkorn et al., 1994). HXGPRT is a purine salvage enzyme that has previously been shown to serve as a useful marker for either positive or negative selection by using MPA or 6TX respectively (Donald et al., 1996). Mammalian host cells have the analogous mammalian enzyme, hypoxanthine-guanine phosphoribosyltransferase (HGPRT) that can not recognize the xanthine analogs such as 6TX. HXGPRT is not an essential enzyme in *Toxoplasma*, because AMP and IMP are interconvertible (Donald et al., 1996; Pfefferkorn et al., 1994).

1.1.4 Molecular tools for down-regulation of gene expression in *T. gondii*

A wide variety of molecular tools, such as insertional mutagenesis and RNA-based gene modulators, have been developed for the study of gene expression knock-down in *T. gondii* system. (Roos et al., 1994; Boothroyd et al., 1994; Black et al., 1995; Meissner et al., 2001; Striepen et al., 2002; Nakaar et al., 1999, 2000; Al-Anouti and Ananvoranich, 2002, 2003).

1.1.4.1 Gene knock-out by insertional mutagenesis

Insertional mutagenesis is quite feasible in *T. gondii* (Donald and Roos, 1995; Donald et al., 1996). The transformants can be screened by applying a selectable marker. To knock out *UPRT* gene by insertional mutagenesis, Donald and Roos (1995) constructed a plasmid that consisted of a DHFR-TS minigene. The disrupted *UPRT* gene conferred resistance to FDUR. Consequently, the transformants were selected by the FDUR.

Donald et al. (1996) first identified the parasite *HXGPRT* gene by insertional mutagenesis. Cells were transfected with a mutated DHFR vector and selected for resistance to 6-TX and sensitivity to MPA. The activity of UPRT and HXGPRT of the knock-out transformants could be restored by transfecting those FDUR/6TX-resistant transformants with reconstructed minigenes encoding the functional UPRT and HXGPRT genomic loci.

1.1.4.2 RNA-based gene modulators

RNA tools, such as antisense and ribozyme, have been developed in *T. gondii* (Nakaar et al., 1999, 2000; Al-Anouti and Ananvoranich, 2002, 2003). As applied in the other gene therapy field (Green et al., 1986), antisense had been used to inhibit *T. gondii* proliferation. Nakaar et al. (1999) applied an antisense NTP RNA construct in which the 3'-untranslated region is replaced by a hammerhead ribozyme to target nucleoside triphosphate (NTPase) gene expression. A dramatic reduction in the steady-state levels of NTPase accompanied by the reduction in parasite replication was observed. The possibility and feasibility of using antisense RNA technology to develop a stable transformation system in *T. gondii* has been demonstrated (Nakaar et al., 1999, 2000). The RNA tools circumvents the shortage of low frequency of homologous recombination in *T. gondii* and provides a new way to investigate the biological function of essential genes. Antisense and delta ribozyme had been tested to interrupt the UPRT parasite nucleotide salvage pathway and the results showed that either engineered delta ribozyme or double-stranded RNA down-regulate the expression of *UPRT* (Al-Anouti and Ananvoranich, 2002, 2003). In these experiments, one delta ribozyme was engineered and used in comparison to antisense RNA and

dsRNA. There was no detailed construction and characterization of delta ribozyme was reported

1.1.5 *T. gondii* and the development of antiparasitic regimens

T. gondii is normally controlled by the immune system of healthy individuals (Pfefferkorn and Pfefferkorn, 1980). It can also be treated by antibiotics or vaccine. However, treatment regimens, for the infection are not ideal due to the complications of treating congenital infection and high incidence of toxic and allergic reaction during chronic treatment of immuno-deficient patients (Luft et al., 1992). Thus the search for new and effective drugs against these pathogens has escalated over the last several years.

Gene therapy provided a new way to control this parasite (Roos, 1996). *T. gondii* can be used to study the potential targets for antiparasitic drug development. The possibility of generating null mutants by gene knock out in *T. gondii* enables the study of gene function that can lead to the identification of potential drug targets. Both UPRT and HXGPRT provide a useful model for the study of the efficiency of the molecular tools. Recent approaches that aim at down-regulating the expression of the genes required for the parasite's invasion and growth by using molecular tools seem very promising. The current challenge is to study the effect of the molecular genetic tools that down-regulate target genes in *T. gondii* and furthermore apply the efficiency tools to interrupt the basic metabolic pathways in *Toxoplasma*.

1.2 Ribozymes

Ribozymes, ribonucleic acid (RNA) enzymes, are catalytic RNAs that can catalyze some necessary reactions in the cells (Jaeger, 1997). The speculation that

RNA could be an enzyme was first given by Francis Crick and others in the 1960s (Jaeger, 1997). Fifteen years after, Cech (Kruger et al., 1982) and Altman (Guerrier-Takada et al., 1983) provided the experimental evidence which supported this speculation by identifying group I introns and ribonuclease P (RNase P), respectively. Cech and his colleagues found that the ribosomal RNA precursor from *Tetrahymena thermophila* contained an intron (group I intron), interrupting the coding sequence of an rRNA molecule, that was capable of excising itself. Altman and colleagues discovered that the RNA subunit of RNase P in *Escherichia coli* and *Bacillus subtilis* is a natural enzyme that is able to process the hydrolytic cleavage of the 5' termini of tRNA precursors without any protein factors. These discoveries established that ribozymes are indeed biological catalysts. Since then, several ribozymes have been identified and subsequent research has led to the characterization and application of these ribozymes.

1.2.1 Naturally occurring ribozymes

Ribozymes have been widely found in nature, such as plants, bacteria, viruses, and lower eukaryotes (Tanner, 1999; Symons 1997). According to their size and reaction mechanisms, the known ribozymes are classified into two groups: the small ribozymes and large ribozymes. The small ribozymes, which range in size from about 35 to about 155nt, consist of hammerhead, hairpin, hepatitis delta ribozyme, and *Neurospora* VS ribozyme. Hammerhead, hairpin, hepatitis delta ribozyme, were named according to their difference in their secondary structures and they cleave RNA during the rolling-circle replication of various viroids and satellite viruses (Sharmeen et al., 1988; Hampel and Tritz, 1989). The lately characterized small ribozyme, *Neurospora* VS ribozyme, appears to play a role in the cleavage of

Neurospora VS RNA, a mitochondrial satellite (Knnell et al., 1995). Large ribozymes including Group I self-splicing intron (Kruger et al., 1982), RNase P (Guerrier-Takada et al., 1983), and group II self-splicing intron (Michel et al., 1995), consist of sizes from a few hundred nucleotides to around 3000 nt. Group I and group II intron are capable of excising itself and RNase P can cleave the 5' termini of tRNA precursors.

1.2.2 Mechanisms of ribozymes

Despite their diversity, all known classes of naturally occurring ribozymes catalyze RNA cleavage reactions via nucleophilic attack on the phosphodiester backbone of RNA. The small ribozyme group, which includes hammerhead, hairpin, hepatitis delta ribozymes, and *Neurospora* VS ribozymes, use 2'-oxygen of the ribose as a nucleophile to attack the adjacent phosphorus and generate products with a 2', 3' cyclic phosphate and a free 5' hydroxyl termini. The large ribozymes, RNase P, Group I and Group II introns catalyze reactions by means of two sequential *trans*-esterifications. These concerted cleavage-ligation reactions alter the phosphate backbone of RNA and generate products with 3' hydroxyl and 5' phosphate termini (Tanner, 1999; Doherty and Doudna, 2000).

Unlike protein ribonucleases, ribozymes cleave only at a specific site and the specificity is determined by the Watson-Crick base-pairing between ribozyme and its target mRNA. The high specific binding and affinity between complementary sequences enable the reactions catalyzed by ribozymes in biological systems with very high precision. Although the rate enhancement of ribozyme is much less compared with protein enzyme, their efficiency is much higher because ribozymes can function directly to mRNA (Narlikar et al., 1997).

1.2.3 Ribozyme engineering

The development of biotechnology made it possible to engineer new catalytic RNA molecules with altered substrate specificity but without changing their activity from the known ribozymes. For instance, several *cis*-cleaving ribozymes have been engineered to *trans*-acting ribozymes that cleave the chosen target RNA. By *in vitro* modification, group I introns can catalyze cleavage in *trans*-acting ribozymes, from a single excision to catalyze multiple turnover (Rossi et al., 1998). The *in vivo* experiment showed that the improved *trans*-splicing ribozyme are active with high efficiency (Kohler et al., 1999). Bartel and Szostak (1993) demonstrated that the substrate specificity of a *trans*-acting hammerhead ribozyme is changed by changing the nucleotides that base pair with the substrate. Several *trans*-acting delta ribozymes have been constructed and they showed to be promising in practical applications (Been & Wickham, 1997).

1.2.4 Applications of ribozymes

Scientists recently paid a lot of attention to the investigation of ribozymes with potential clinical applications (Chowrira et al., 1994; Welch, et al., 1998). Ribozymes are currently being developed as inhibitors of gene expression and viral replication. The application of ribozymes in mammalian cell systems includes gene expression knockdown, RNA repair, and gene functional analyses (Rossi, 1999).

The primary application of ribozymes was on anti-virals, (such as HIV, hepatitis B virus, hepatitis C virus) since they have no DNA intermediates. For example, hairpin and hammerhead ribozymes delivered by retrovirus were used to inhibit HIV viral replication in T cells and CD34⁺ stem cell progeny (Ramezani et al., 1997; Gervais et

al., 1997). Ribozymes have also been used to target cellular genes, which become a highly potential weapon against cancer. Successful examples of sequence specific inhibition of gene expression in cell culture have been found using hammerhead ribozymes to target the mRNA of oncogenes e.g. *bcr-abl* (Kuwabara et al., 1998), *ras* (Kijima et al., 2000; Tokunaga, et al., 2000). For example by targeting *bcl-2* with ribozymes, cancer cells can be triggered to undergo apoptosis (Dorai et al., 1997). Inhibition of interleukin-1beta (IL-1beta) production in human cells by hammerhead ribozymes against IL-1beta and IL-1beta converting enzyme was studied by Leavitt, et al. (2000). AAV-vectored ribozymes have been used to treat autosomal dominant retinitis pigmentosa in animal models of this blinding disease and provided significant protection of functional vision (Lewin et al., 2001).

Group I intron can repair a defective mRNA by catalyzing *trans*-splicing. Watanabe and his coworkers used group I intron to repair p53 and sickle-cell hemoglobin mRNA in culture cells (Watanabe et al., 2001). Group II intron RNA can form a complex with an intron-encoded endonuclease which catalyzes the cleavage of DNA at the target site for transposition. Guo et al. (2000) used a group II intron from *Lactococcus lactis* to disrupt sites in the HIV-1 provirus and the gene encoding the chemokine receptor in mammalian cells. Delta ribozymes have been applied in the synthesis of RNA with the precise termini required in the production of defective interfering particles of vesicular stomatitis virus (Chowrira et al., 1994). In a recent study, Schurer et al. (2002) demonstrated that delta ribozyme can be used to overcome the drawback of *in vitro* transcription.

As ribozymes can down-regulate specific gene expression, they can help to identify the function of a protein or the role of a gene in a functional cascade. By introducing a ribozyme against matrix metalloproteinase-9, the role of

metalloproteinase-9 in prostate carcinoma metastasis can be verified (Sehgal et al., 1998). While ribozymes are used in pathway elucidation and target validation, they are promising tools to discover new genes that are involved in a particular phenotype (Welch et al., 1998).

The major difficulties for gene suppression agents lie in an effective cellular delivery and target colocalization. In most instances, ribozymes have been delivered by vectors based on molony murine leukemia virus (MMLV) (Amado et al., 1999; Looney et al., 1997; Copper et al., 1999). Adeno-associated virus (AAV) vectors (Hernandez et al., 1999) have also been used. Ribozyme expression can be restricted to specific organs or cell types through the use of tissue-specific promoters (Ohla et al., 1996). Ribozymes normally colocalize in the cytoplasm to target the transcript mRNA, although tRNA-driven ribozymes have been detected in the nucleus (Bertrand et al., 1997).

It is possible to generate chemically stabilized ribozymes through either modifying phosphate or 2'-hydroxyl groups (Beigelman, 1995). However, that might bring other problems, such as the stability and activity of the synthetic ribozymes in living cells. The endogenous synthesis ribozymes appears to be an attractive tool in ribozyme-based therapy. RNase P, an endogenous ribozyme, can be used to digest cellular or viral mRNAs without the trouble in delivery because it can be synthesized constitutively in cells (Altman, 1995). Guerrier-Takada et al. (2000) have used RNase P to block the expression of marker genes in bacteria and destroy influenza virus transcript in mammalian cells.

Although great progress has been made in the field of ribozymes, there are still problems that involve the extra- and intracellular stability of the engineered ribozyme, target accessibility, optimal catalytic activity and specificity of the ribozyme.

1.3 Delta ribozymes

Hepatitis delta virus (HDV) is a human pathogen that requires hepatitis B virus (HBV) for its life cycle. It can cause severe fulminant hepatitis in infected patients. The genome of HDV contains a circular RNA (about 1700 nucleotides). The replication of both genome and its complementary antigenome of HDV involves self-cleavage activity facilitated by two highly conserved RNAs with ribozyme activities that process the linear multimeric RNA into monomeric RNAs. These two 84 nt conserve motifs are named delta ribozyme. One is on the genomic RNA strand that is called genomic delta ribozyme, while the other in the complementary region of the antigenomic strand is called antigenomic delta ribozyme (Taylor, 1991, 1992; Symons, 1992; Been, et al., 1994).

1.3.1 Secondary structure of delta ribozymes

Despite the sequence differences, both the genomic and antigenomic forms of the delta ribozyme have similar secondary structures as shown in Figure 1.1 (Perrotta and Been, 1991; Rosenstein and Been, 1991, Shih and Been, 2002). The secondary structure of delta ribozymes consists of one stem (P1), one pseudoknot (P2), two stem-loops (P3 and P4), L3 loop, and three single-stranded junctions referred to as the linker stems (J1/2, J1.1/4 and J4/2). In addition, a second pseudoknot, helix P1.1, was formed by two GC base pairs from residues of the P3 loop and J1.1/4 junction in delta ribozyme secondary structure that has been confirmed by X-ray crystallographic studies (Ferré-D'Amare et al., 1998). Crystal structure of genomic and antigenomic forms of delta ribozymes have been computer modeled and provided views of the tertiary folds of these RNA (Tanner et al., 1994; Shih & Been 2002).

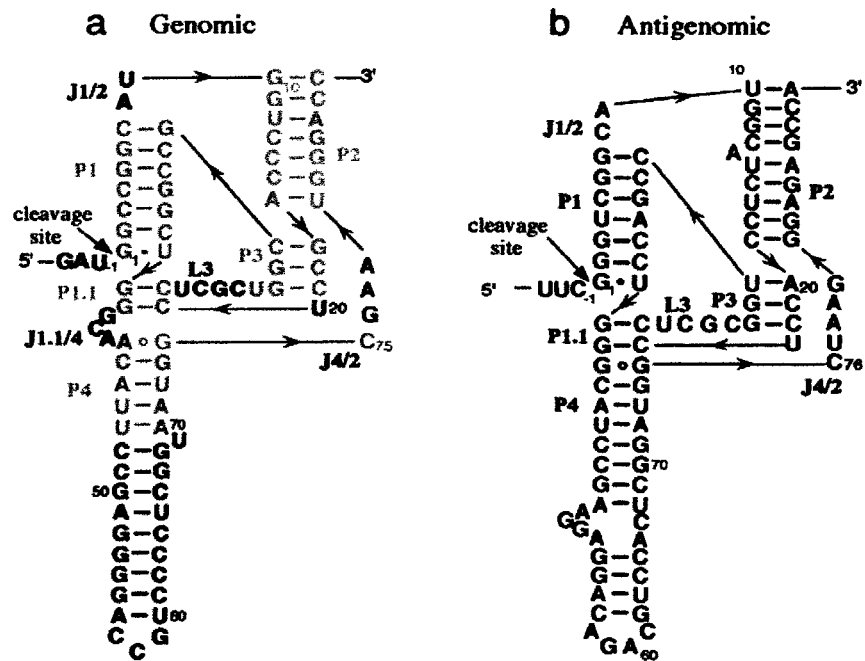
FIGURE1.1

Sequence and Secondary Structure of the HDV Ribozymes

Legend

Sequence and secondary structure of wild-type genomic HDV ribozyme (a) and antigenomic HDV ribozyme (b). P1-P4 and P1.1 base-paired stems, L3 loop, and J1/2, J1.1/4, J4/2 joining regions between two helices are shown. The cleavage site is also pointed (modified from Shih and Been, 2002)

FIGURE 1.1



1.3.2 Catalysis of delta ribozyme

Like other small catalytically active ribozymes mentioned at the beginning, both genomic and antigenomic HDV ribozymes cleave a phosphodiester bond of their RNA substrates, yielding products with 5'-hydroxyl and 2',3'-cyclic phosphate termini. Delta ribozymes are metalloenzymes. The cleavage reactions occur in the presence of divalent metal cations, such as Mg^{2+} , Mn^{2+} and Ca^{2+} , under physiological pH conditions (Kuo et al., 1988; Sharmeen et al., 1988). The presence of Mg^{2+} is essential for the cleavage. Evidence from Ananvoranich and Perreault showed that addition of $MgCl_2$ causes HDV ribozyme a structural change in the folding of the L3 loop during the cleavage reactions (Ananvoranich & Perreault, 2000).

The sequence of an RNA molecule will determine the folding and the sequential folding kinetics of the ribozyme might directly regulate its *in vivo* function (Isambert & Siggia, 2000). A correlation between the folding and catalytic behavior of delta ribozymes was demonstrated (Ananvoranich & Perreault, 2000) using hybridization of complementary oligo deoxynucleotides and RNase H hydrolysis. Substrate recognition requires formation of the P1 stem, comprising a GU wobble pair plus six nucleotides hybridized to the target. The cleavage occurs just 5' to the wobble base pair (Jeng et al., 1996; Wadkins et al., 1999).

1.3.3 *Trans*-acting delta ribozymes

Like other ribozymes, the *cis*-cleaving activity of the delta ribozyme can be converted into a *trans*-cleaving activity (Tanner, 1995; Been et al., 1997; Lazinski and Taylor, 1995). The 57 nt *trans*-cleaving delta ribozymes (Fig. 1.2) have been designed that are active against oligonucleotide substrates by removing a junction between P1 and P2 stem and shortening the P2 and P4 stem from the antigenomic delta ribozyme.

Thus, the substrate sequence is separated from the catalytic domain and the intramolecular self-cleaving activity is converted into a *trans*-cleaving activity (Ananvoranich & Perreault, 1998). The activity of this engineered *trans*-acting delta ribozyme has been well supported by experiment data (Roy, et al., 1999). The specificity of *trans*-acting antigenomic ribozyme was investigated with substrates that varied in either the length or the nucleotide sequence of their P1 stems (Ananvoranich & Perreault, 1998; Ananvoranich et al., 1999; Deschenes et al., 2000).

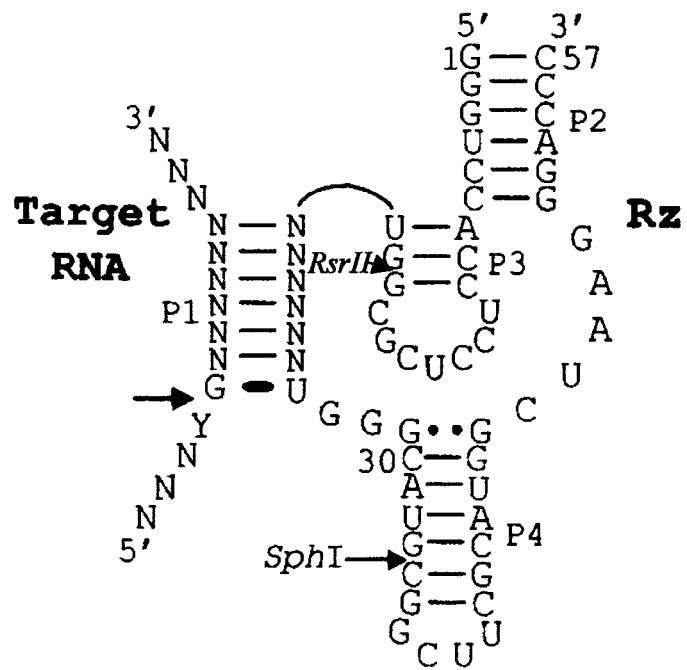
FIGURE 1.2

Secondary Structure of Engineered *Trans*-acting Delta Ribozyme Derived from the Antigenomic Self-cleaving Motif

Legend

Four stems (P1-P4) are shown. Y and N represent a pyrimidine and any nucleotide (G, A, U, C), respectively. In the target RNA substrate, the arrow points to the cleavage site. In the ribozyme (RZ), the arrow indicates the *SphI* and *RsrII* restriction sites used for modification of the ribozyme binding sequence (modified from Roy et al., 1999).

FIGURE 1.2



1.3.4 Characteristics of delta ribozymes

Delta ribozymes are among the best-characterized small RNAs. Like other naturally occurring ribozymes, they are able to specifically recognize and bind to a target RNA molecule through Watson-Crick base pairing and then catalyze its cleavage. Moreover, delta ribozymes possess several unique features compared with other ribozymes. A major advantage of delta ribozymes lies in the fact that it is the only catalytic RNA known to be naturally active in human cells.

Unlike other small ribozymes, delta ribozyme can function at low concentrations of Mg^{2+} (about 1 mM) and it can be fully active in the presence of calcium, under these human-like physiological conditions (Doherty & Doudna, 2000). Since this ribozyme functions in human cells, it is also active in the presence of human proteins. Also it will not cause any immunogenicity problems that other reagents may cause (Levesque et al., 2002).

Delta ribozymes are one of the fastest naturally occurring ribozymes. The self-cleavage rate of delta ribozyme is about 100 times faster than that of the hammerhead (Wu et al., 1990; Thill et al., 1993).

Delta ribozymes also have an outstanding stability after cell transfection (Levesque, et al., 2002). In delta ribozyme, both the 5' and 3' ends are located within the P2 stem, which appears to greatly stabilize the molecule (Doherty & Doudna, 2000). Although the tested delta ribozyme appears only slightly more stable than a hammerhead ribozyme in cell-free protein extract, it exhibits quite stable in the transformed cells with half-life of over 100 hours (Levesque et al., 2002). As an endogenous ribozyme, delta ribozymes can exist stably long enough for their function and do not need cost to have any modification. Thus, it is a preferable tool to be used in gene therapy.

All the above unique properties make delta ribozymes distinguish from other ribozymes and determined that delta ribozymes could be engineered to a gene therapeutic tool which could inactivate mRNAs associated with human disease (Sun et al., 2000, Lai, 1995).

Objectives of this study

The initial goal of this study was to construct delta ribozymes and study the cleavage activity of these constructed delta ribozymes *in vitro*. The second objective was to demonstrate the activities of the engineered delta ribozymes in *T. gondii*. The stable cell line of *T. gondii* obtained by electroporating the plasmids with cDNA of the engineered ribozyme and selecting in drug FURD were characterized at the end.

CHAPTER 2

MATERIALS

2.1 Chemicals and supplies

2.1.1 Enzymes

Restriction enzymes *Bam*HI, *Sac*II, and *Sph*I were obtained from Promega (Madison, WI). Restriction enzymes *Sma*I and *Eco*RI were from MBI Fermentas (Burlington, ON). Restriction enzymes *Rsr*II and *Pvu*II were purchased from New England BioLabs (Beverly, Mass). Proteinase K, and RNase out ribonuclease inhibitor were from Invitrogen Corp. (Burlington, ON). RNase A, and T4 polynucleotide kinase were from USB (Cleveland, OH). *Taq* DNA polymerase was purchased from Roche Diagnostics (Laval, Quebec). Mung Bean Nuclease, T4 DNA ligase, Moloney murine leukemia virus reverse transcriptase (M-MLV), and RQ1 RNase-free DNase were obtained from Promega (Madison, WI). Calf intestinal alkaline phosphatase (CIAP) and T7 polymerase were from Amersham-Pharmacia Biotech (Baie d'Urfe, Quebec).

2.1.2 Media

Cell culture media Dulbecco's modified medium (DMEM), minimal essential medium (MEM), phosphate buffer supplemented with calcium, trypsin, penicillin-streptomycin (10 u/mL) and L-glutamine were all from GibcoBRL Invitrogen (Burlington, ON). 10% Cosmic calf serum was from Hyclone (Logan, UT) and 1% dialyzed fetal bovine serum was from Wisent (Montreal). Tryptone, yeast extract, and agar used for making bacteria culture media LB broth or LB plates were from Difco. Ampicillin and Tetracycline were from Sigma.

2.1.3 Other chemicals and supplies

The PicoGreen dsDNA Quantitation Kit and lambda DNA standard was from Molecular Probes (Eugene, OR). The Midi-prep plasmid purification kit was from Sigma. The T₇ Sequencing Kit was from USB (Cleveland, OH). Agarose, DNA ladders (1 kb and 100 bp) were purchased from Promega (Madison, WI). Acrylamide, bis-acrylamide, ammonium persulfate, urea, equilibrated phenol (pH > 8), and sodium dodecyl sulphate (SDS), glycerol were from USB (Cleveland, OH). Bromophenol Blue, Xylene Cyanol, and Bio-Rad protein assay reagent were obtained from Bio-Rad Laboratories (Mississauga, Ontario). SYBR-gold was from Molecular Probes. Digoxigenin-11-dUTP (DIG-dUTP), anti-digoxigenin alkaline phosphatase Fab antibody, blocking reagent, and CSPD chemiluminescent substrate were from Roche Diagnostics (Laval, Quebec). 2'-deoxyribonucleoside 5'-triphosphates (dNTPs), ribonucleoside triphosphates (rNTPs), Hybond N⁺ Nylon membranes, [³²P]-dATP (3000 Ci/mmol), and Sephadex G-25 were from Amersham-Pharmacia Biotech (Baie d'Urfe, Quebec). [³H] uracil and [³H] hypoxanthine monohydrogen chloride were obtained from Perkin Elmer (Norwalk, CT). NalgeneTM disposable 25 mm syringe filters (0.2 µm pore size) and glass Pasteur pipettes were from Baxter Diagnostics Corp. (Toronto, ON). Conical 15 mL and 50 mL graduated polypropylene centrifuge tubes, disposable pipette tips, 24 well plates, Petri dishes, Tissue culture dish (60 x 15 mm), cell scrapers, 13 mL loose cap culture tube, and T75 and T25 tissue culture flasks, Falcon tubes were supplied either by VWR (West Chester, PA) or by Sarstedt Incorp.(Newton, NC, USA). Polycarbonate filters (3 µm pore size), were obtained from Whatman Nucleopore, ON. Microtube (1.5 mL) was from Ultident.

Glutathione, Polyoxyethylene sorbitan monolaurate (Tween-20), dithiothreitol (DTT), ethidium bromide, sodium citrate, sodium chloride, sodium acetate, ethanol, ethylenediaminetetra-acetic acid disodium salt, trishydroxymethylaminomethane (Tris), 2-propanol, glacial acetic acid and hydrochloric acid, HEPES, FDUR prodrug, dimethylsulfoxide (DMSO), diethylpolycarbonate (DEPC), Trichloroacetic acid (TCA), scintillation fluid, and RNA grade formamide were obtained from Sigma-Aldrich (Oakville, Ontario). CaCl_2 , Tris base, was from Fisher Scientific. MgCl_2 , NaOH, NaOAC, K_2HPO_4 , KH_2PO_4 , KCl, chloroform, and glucose were from BDH. EDTA, Trizol, and TEMED were from GibcoBRL. All other chemical and supplies not mentioned were either from Sigma or BDH (Toronto, ON). All oligonucleotides listed in Appendix Table were synthesized by Sigma Genosyn.

2.2 Apparatus and instrumentation

Absorbance measurements for RNA and DNA quantification were carried out by using the Agilent UV-visible spectrophotometer from Agilent Technologies (Mississauga, ON). The quartz cuvettes were from Sigma. The SPECTRAMax GEMINI XS dual-scanning microplate spectrofluorometer from Molecular Devices Corp. (Sunnyvale, CA) with Soft max[®] PRO software was also used for the determination of DNA concentration following PicoGreen staining. Polymerase chain reactions (PCR) were performed using either the 48-well Perkin Elmer Cetus DNA thermal cycler (Perkin Elmer, Norwalk, CT) or the 20-well Techgene thermal cycler (Techne, Cambridge, UK). Agarose gel electrophoresis of DNA and RNA was performed using the Miniature Horizontal Gel System MLB-06 from Tyler Research Instruments (Edmonton, AB). The gels were

viewed using the Benchtop Ultraviolet Transilluminator supplied by VWR Scientific (Mississauga, ON). Gel images were viewed and captured by the AlphaImager™ 2200 Light Imaging System with AlphaEase software for photography of ethidium bromide stained agarose gels from Alpha Innotech Corporation (San Leandro, CA). UV irradiation shelter was from Ultra-Violet Product Inc. (San Gabriel, CA).

Polyacrylamide gels were carried out by using the vertical gel electrophoresis system, including all glasses, Teflon comb and spacers, from BRL (Bethesda Research Laboratories). Centrifugations were carried out in either the J2-HS Centrifuge (Beckman) or the desktop Eppendorf Model 5415C microcentrifuge from Desaga (Sarstedt Gruppe, Germany). The DNA was transformed from gel to membrane using a vacuum blotter (Boekel Scientific, PA), and the blots were UV crosslinked using the CL-1000 UV crosslinker (Upland, CA). Hybridization assays were performed using either the ProBlot hybridization oven obtained from Labnet (Mandel) or the Bambino hybridization oven (Boekel Scientific, PA).

Cell and parasite culturing was conducted under sterile conditions in the Class II type A/B3 Biosafety cabinet (Jouan, SA), and all cultures were maintained in a CO₂ incubator (Thermo Forma). All electroporations were performed using the BTX model 600 Electro Cell Manipulator supplied by Genetronics (San Diego, CA) and the electroporation cuvettes were also from the same company. Radioactivity was measured by a Scintillation counter model LS-6500 (Beckman, Fullerton, CA). The imaging screens used for gel exposure were scanned by Cyclone Imaging system (Canberra Packard). Other general laboratory equipment used included the following:

A Corning pH Meter 340 (Corning, NY) with standardized buffer solutions from BDH Inc. (Toronto, ON); a Sartorius BP61 balance; a Corning Stirrer; a Genie 2 Vortex from Fisher Scientific (Toronto, Ontario); Eppendorf pipettes (Germany); Freezer vials (VWR); a Standard Heatblock (VWR); a C25 incubator shaker (New Brunswick Scientific Classic Series, NJ), and a Size 2 Economy Incubator (Gallenkamp).

CHAPTER 3

METHODS

3.1 Engineering of delta ribozymes

3.1.1 Selection of target sites

The entire open reading frame of *UPRT* and *HXGPRT* cDNA sequence of *Toxoplasma gondii* was downloaded from the gene bank (<http://www.ncbi.nlm.nih.gov>) (Gene Bank No. U10246 and U10247, respectively). The secondary structures of *UPRT* and *HXGPRT* cDNA were predicated by free energy minimization using Zuker's mfold programTM (version 3.1; <http://bioinfo.math.rpi.edu/~zukerm/rna/node3.html>; Zhao et al., 1998). The conserved single-stranded regions in *UPRT* mRNA and *HXGPRT* mRNA were chosen for the target sites.

3.1.2 Construction of plasmids

3.1.2.1 Plasmids constructing

The *trans*-acting delta ribozyme described by Roy et al. was used in delta ribozyme engineering (Fig. 1.2, Roy et al., 1999). The recognition sequence (7 nt) located in the P1 stem was modified to specifically complimentary to the chosen target *UPRT* and *HXGPRT* mRNA sequence. The pair of synthesized upper and lower oligonucleotides (sequences were listed in Appendix Table) for each ribozyme were annealed by heating at 95°C for 2 min and the reactions were left cool down slowly at room temperature. Then the annealed oligonucleotides for each ribozyme were ligated into the digested minigene respectively. The ligation reactions contained 1 unit of T4 DNA ligase, 30 mmol/L Tris-HCl (pH 7.8), 10 mmol/L MgCl₂, 1 mmol/L ATP and 10 mmol/L DTT and were

incubated at 16°C overnight. The resulting four different plasmids with delta ribozyme cDNA were named pUC19-UPRz1, pUC19-UPRz2, pUC19-UPRz3, pUC19-UPRz4, pUC19-HXRz1, and pUC19-HXRz2.

3.1.2.2 Transformation of *E.coli* with constructed plasmids

A half of the ligation reaction (10 µL) were mixed with 25 µL competent *E. coli* cells. After the tubes were incubated on ice for 20 minutes, the cells were heat-shocked for 45 sec at 45°C using a Standard Heating block, and incubated on ice for another 2 minutes. After the addition of 400 µL LB broth, the transformed bacteria were incubated at 37°C with shaking at 225 rpm for 45min. Subsequently, the culture (100 µL) were spread onto LB agar plates containing 0.1g/L ampicillin. Agar plates were incubated overnight at 37°C.

3.1.2.3 Isolation of the constructed plasmids from *E.coli* (miniprep)

Single isolated colonies on the overnight growth LB agar plates were inoculated to sterile loose-capped culture tubes containing 3 mL of LB broth supplemented with 0.1 g/L ampicillin, and then grew in incubator with shaking at 37°C overnight. The cultures were pelleted by centrifuging at 12,000 rpm for 1 minute and the subsequent pellets were resuspended in 100 µL of an ice-cold lysis buffer containing 50 mM glucose, 25 mM Tris-HCl (pH 8.0), and 10 mM EDTA. Following 200 µL of freshly prepared neutralization buffer containing 0.2 N NaOH, 1% SDS was added to the mixture and incubated at room temperature for 5 minutes, 150 µL of ice-cold 3 M sodium acetate, pH 5.2 was added, and the reaction was vortexed, incubated on ice for 5 minutes. To pellet

the cellular debris, the mixture was centrifuged at 12,000 rpm for 5 minutes and then the clear supernatant was transferred to a new sterile microcentrifuge tube. Equal volume of Tris EDTA buffered phenol:chloroform:isoamyl alcohol (25: 24: 1) was added to the supernatant extract, and the mixture was vortexed and centrifuged at 12,000 rpm for 1 minute to separate the organic and aqueous layers. The top aqueous layer in which the constructed plasmids contained was transferred to a fresh tube and was precipitated with two volumes of 95% ethanol. After the subsequent mixture was incubated at -20°C for 10 minutes, it was centrifuged at 12,000 rpm for 15 minutes at 4°C. The resulting pellet was washed with cold 70% ethanol, centrifuged, air dried and resuspended in 30 µL of Tris EDTA buffer (10 mM Tris, 1 mM EDTA, pH 8.0) containing 20 µg/mL RNase A, and incubated at 37°C for 15 minutes to degrade RNA. All the extracted plasmid samples were stored at -20°C for future use.

3.1.3 Confirmation of the constructed plasmid

3.1.3.1 Restriction enzyme digestion

The constructed plasmids were checked first by digestion with *Rsr*II at 37 °C overnight. Then the digestion reaction was subjected to 1% agarose gel electrophoresis.

Miniature Horizontal Gel System was used for agarose gel electrophoresis. 1% (w/v) agarose gels were prepared by dissolving solid agarose in TAE buffer (40 mM Tris-acetate, 1 mM EDTA) followed by adding 5 ng/mL of ethidium bromide. Following solidification, the gel was immersed in TAE buffer. DNA samples mixed with gel loading buffer (6x: 0.25% (w/v) Bromophenl Blue, 0.25% (w/v) xylene cyanol FF, and 40 %(w/v) sucrose in water) were loaded into the wells along side a 1 kb marker. The

agarose gel was electrophoresed for about 0.5 - 1 hour at 100 volts. It was then viewed and photographed using the AlphaImager 2200 Light Imaging System with AlphaEase software.

3.1.3.2 Sanger dideoxynucleotide sequencing

The sequence of the plasmid construction was confirmed by DNA sequencing using T₇ Sequencing Kit. Briefly 10 pmol universal or reverse primer was quickly annealed to 2 µg constructed plasmid with represent 0.13 M NaOH (1 M NaOH 1.5 µL in total 11.5 µL reaction volume) by incubating at 65°C for 5 min. Equal amount of HCl and annealing buffer containing 1 M Tris-HCl (pH 7.5), 100 mM MgCl₂ and 160 mM DTT were added and incubated at 37°C for 10 min at room temperature for another 5 min. Labeling reaction with labeling mix-dATP (1.375 µM each dCTP, dGTP and dTTP and 333.5 mM NaCl) and ³²P labeled r-dATP and T₇ DNA polymerase was prepared and added to each of the four pre-warmed sequencing mix (“A” Mix-short, “C” Mix-short, “G” Mix-short, “T” Mix-short, supplied by the kit). Following incubation at 37°C for 5 min, stop solution (97.5% deionized formamide, 10 mM EDTA, pH 7.5, 0.3% bromophenol blue, 0.3% xylene cyanol) was added. After denatured at 80°C for 2 min, samples were run on 5% denaturing polyacrylamide gel.

A stock solution containing 19% (w/v) acrylamide, 1% (w/v) bisacrylamide, 8 M urea, and Tris Borate EDTA (TBE) was used to make different concentrations of polyacrylamide gel polymerized by adding 450 µL 10% ammonium persulfate and 35 µL TEMED in a 40 ml gel solution. The gel solution was poured between the gap of the two glass plates separated by the spacer. After polymerization (about 1 hour), the denatured

samples were loaded and the gel was run in 0.5 x TBE buffer at about 800 volts. Subsequently, the gel was exposed to the imaging screen over night at 4°C and the screen was scanned with the Cyclone Imaging system.

3.2 Cleavage activity of engineered delta ribozymes *in vitro*

3.2.1 Synthesis of delta ribozymes

3.2.1.1 Large scale plasmid preparation by Midiprep

After the sequence of each ribozyme was confirmed, their purified coding plasmids were extracted from the overnight LB broth culture containing ampicillin using the Sigma Gen-elute Plasmid Midi-Prep Kit as the manufacturer's directions. The purified plasmids were frozen in -20°C for future use in synthesis of ribozymes and electroporation. The subcultures of *E.coli* with constructed plasmids were frozen in -80°C with 30% glycerol.

3.2.1.2 Synthesis of delta ribozymes by *in vitro* transcription

Ten microgram of each constructed plasmid, pUC19-UPRz1, pUC19-UPRz2, pUC19-UPRz3, pUC19-UPRz4, pUC19-HXRz1, and pUC19-HXRz2 was digested with *SmaI* in 50 µL reaction at 30°C overnight. The digestion result was checked on 2% agarose gel and then the digestion reactions were phenol extracted, ethanol precipitated, and dissolved back into 50 µL. Five microliter of the extracted digestion reaction was used for the synthesis of ribozymes by *in vitro* transcription. The transcription was performed overnight at 37°C in a 50 µL reaction containing 2 mM DTT, 50 units RNase inhibitor, 5 mM rNTP, 200 units T7 RNA polymerase, and 1/100 dilution pyrophosphatase in the buffer with 80 mM HEPES-KOH, 24 mM MgCl₂, 2 mM

spermidine. In order to get rid of the DNA left in the reaction, two units of RNase free RQ1 DNase was added and further incubated at 37°C for 30 min. After extraction with phenol, the products were separated on a 10% polyacrylamide gel that contained 8 M urea. Following visualization by UV irradiation shelter, the appropriate band was excised from the gel and eluted with elution buffer (0.5 M ammonium acetate, 0.1% SDS) over night. Subsequently, the RNA was recovered by precipitation with 0.1 volume of sodium acetate (pH 5.3), two volume of 100% ethanol and quantified by UV spectrophotometer.

3.2.2 Delta ribozyme cleavage reaction using model *UPRT* mRNA substrates (15nt)

3.2.2.1 Synthesis of model *UPRT* mRNA substrates

Oligonucleotide (15 nt, 500 pmol) with each of the target *UPRT* mRNA sequence (ComSub_1_15_long, ComSub_2_15_long, ComSub_3_15_long, ComSub_4_15_long) and PST T7 Oligo (500 pmol) with T7 promoter sequence (Appendix Table) were annealed at 95°C for 2 min and then left cool down slowly at room temperature. Subsequently, the reactions were used as template to synthesize *UPRT* mRNA model substrates by *in vitro* transcription under the conditions described before. After the resulting RNAs were fractioned by electrophoresis on 20% denaturing polyacrylamide gel, the corresponding size band (15 nt) was cut and the RNA was eluted in elution buffer over night. The substrate RNA was precipitated and the concentration was measured by UV spectrophotometer.

3.2.2.2 End labeling of the model substrates

The synthesized model substrates RNAs were dephosphorylated at 5'-end by treatment with 0.2 unit alkaline phosphatase (CIAP) at 37°C for 30 min. Then the dephosphorylated substrates were extracted with phenol, precipitated with ethanol, and finally dissolved in DEPC-H₂O to a concentration of 2.5 pmol/μL. After that, the substrates (2.5 pmol) were 5'-end labeled with ³²P-ATP (10 μCi) in a 15 μL reaction with 5 unit of T4 polynucleotide kinase, 50 mM Tris/HCl (pH 8.0), 10 mM MgCl₂, 5 mM dithiothreitol, 1.7 μM ATP at 37°C for half hour. Then the labeled short substrates were diluted with DEPC-treated water to the final concentration 0.025 pmol/μL and passed through G-25 sephadex to remove the unincorporated free nucleotides. The CPM of the labeled substrates was measured by using a scintillation counter. All the labeled substrates were loaded on 20% denaturing polyacrylamide gel to check for the correct size.

3.2.2.3 Cleavage reaction

The cleavage reaction was performed under single-turnover conditions, i.e. with excess of ribozymes over the substrate. Different amounts of ribozymes (5 nM, 10 nM, 25 nM, 50 nM, 100 nM, 200 nM, 400 nM, 600 nM) were added to 2.5 nM of the corresponding ³²P labeled substrates and the mixtures were denatured in 50 mM Tris-HCl (pH 8.0) at 95°C for 1 min followed by cooling on ice for 1 min. The reactions were then incubated at 37°C for 5 min before the cleavage reactions were initiated by adding 25 mM MgCl₂. The reactions proceeded at 37°C. Aliquots of the reaction mixtures were taken at specified time points and quenched with equal volumes of loading buffer (0.025% xylene cyanol FF, 0.025% bromophenol blue, 98% deionized formamide, 10 mM EDTA) and

separated on 20% denaturing polyacrylamide gel. The gel was exposed to the imaging screen and the screen was scanned with Cyclone Imaging system.

3.2.2.4 Kinetic analyses

The image was quantified using ImageQuant software / PhosphorImager (Cylone). The percentage of substrate cleaved by the ribozymes was calculated with the equation: Percentage of cleavage (%) = (value of cleaved substrate RNA / (value of cleaved substrate RNA + value of uncleaved substrate RNA)) * 100%. The fraction of substrate cleaved was plotted versus time and fitted to single exponential equations. For kinetic analysis, rate of cleavage (k_{obs}) were calculated from the data fitted to a single exponential equation: $[A]_t = [A]_{\infty}(1 - e^{-kt})$, where $[A]_t$ is the fractions cleaved at time t , and $[A]_{\infty}$ is the cleavage reaction end point respectively. The ribozyme activity was plotted with the changing of ribozyme concentration.

3.2.2.5 Cleavage reaction with presence of *T. gondii* cell-free extract

To prepare parasite lysate, two times lysis buffer containing 0.2 mol/L NaOH and 1% SDS (v/v) was added to equal volume of wild type (RH) lysed *T. gondii* parasite pellet from one cell culture plate, about 10^6 parasites/mL, and incubated at room temperature for a few seconds until the mixture became clear. The protein concentration in the lysate was measured by using the Bradford reagent according to the manufacturer's instruction. The unknown concentration of protein was calculated from a standard curve made with standard protein solutions of Bovine Serum Albumin protein, ranging from 1 $\mu\text{g/mL}$ to 10 $\mu\text{g/mL}$. The absorbance of the sample was measured at 595 nm using UV

spectrophotometer. The protein concentration was determined by comparing the A_{595} values against the standard curve.

Effect of WT parasite lysate on the cleavage activity of delta ribozyme was tested under the same conditions as without the lysate. Different amount of the parasite lysate were added to the cleavage reaction containing 250 nM ribozyme and 2.5 nM corresponding labeled substrate. Samples were taken at the reaction starting point and 60 min respectively. The reaction was terminated by equal volume of loading buffer.

Fixed amount of RH parasite lysate was added to the cleavage reaction containing 5 nM, 10 nM, 25 nM, 50 nM, 100 nM, 200 nM, 400 nM, and 600 nM ribozyme respectively and trace amount (2.5 nM) of its corresponding substrate. The reactions were subjected under the same condition as before and aliquots of cleavage reaction were taken at the fixed reaction time point and stopped immediately by adding equal volume of loading buffer. All the above samples were fractioned on 20% denaturing polyacrylamide gel at 500 volts for about 30 min. The gel was exposed on imaging screen overnight and checked with the Cyclone Imaging system.

3.2.3 Primer extension assay

3.2.3.1 Synthesis of *UPRT* mRNA long substrates (150 -720 nt)

Sequences of *UPRT* cDNA down stream 50 - 80 nt of the UPRz1, UPRz2, UPRz3, and UPRz4 cleavage sites were chosen as the reverse transcription primers, named as UPRz1 3'Product, UPRz2 3'Product, UPRz3 3'Product, UPRz4 3'Product (Appendix Table). PCR was carried out to produce different *UPRT* DNA fragments by using oligonucleotide T7on5'UP as forward primer and the above designed reverse

transcription primers as reverse primer respectively. The PCR reaction contained 20 $\mu\text{mol/L}$ of each dNTP, 50 pmol of each primer, and 1 unit of *Taq* DNA polymerase, 20 mmol/L ammonium sulfate, 75 mmol/L Tris-HCl (pH 8.8), 0.1% Tween-20, 2.5 mmol/L MgCl_2 . Five microliter of each 150 nt, 300 nt, 500 nt, and 720 nt PCR product was used individually as the template to synthesize corresponding size of *UPRT* mRNA in a 50 μL *in vitro* transcription reaction. The reaction was performed for 4 hours at 37°C followed by treated with 20 units of DNase for half hour at 37°C. The resulting RNA substrates were then checked on 2% agarose gel.

3.2.3.2 Cleavage reaction of ribozymes using long *UPRT* substrates

The mixture of 12 pmol delta ribozyme with 4 pmol each synthesized 150 nt, 300 nt, 500 nt, 720 nt *UPRT* RNA substrate was denatured in 50 mM Tris-HCl (pH 8.0) at 95°C for 1 min followed by cooling on ice for 1 min. The reactions were then incubated at 37°C for 5 min before the addition of 25 mM MgCl_2 . The reaction was proceed at 37°C for 2 hrs followed by being precipitated with 0.1 volume of 3 M sodium acetate (pH 5.3) and 2 volume of 100% ethanol. The precipitated cleavage reaction was washed with 70% ethanol, air dried and stored in -20°C for reverse transcription.

3.2.3.3 Reverse transcription reaction

RT-primer (UPRz1 3'Product, UPRz2 3'Product, UPRz3 3'Product, UPRz4 3'Product; Appendix Table) that 50-80 nt downstream of each ribozyme cutting site (20 pmol) was annealed to the above corresponding cleavage product at 70°C for 5 min in One-Phor-All Buffer (1x OFA Buffer) consisting of 10 mmol/L tris acetate, 10 mmol/L magnesium

acetate, and 50 mmol/L potassium acetate followed by being chilled on ice for 2 min. The twenty microliter of reverse transcription reactions containing 5 μ L of the above annealed reaction, 20 μ Ci r-*ATP, 0.5 mM of each dNTP, 50 units of RNase out RNase inhibitor, 200 units of M-MLV reverse transcriptase, 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 10 mM DTT, 3 mM MgCl₂ were proceed at 37°C for 40 min and then frozen at -20°C waiting for future electrophorsis with sequencing reaction.

3.2.3.4 *UPRT* cDNA sequencing

UPRT cDNA sequencing was performed using T7 Sequencing Kit as described before. Each of above RT- primer (10 pmoles) were used as sequencing primer, while plasmid pUC18 T7GGG containing *UPRT* insert was used as template. The reverse transcription products were run along with the corresponding denatured *UPRT* sequencing reaction on 5% denaturing polyacrylamide gel. The gel was then exposed to the imaging screen over night and the screen was scanned with the Cyclone Imaging system.

3.3 Down regulation effects of engineered delta ribozymes in *T. gondii*

3.3.1 Host cell culture, subculture and freezing

Human foreskin fibroblasts (HFF; obtained from Dr. D. Roos, University of Pennsylvania) were used as a host for the intracellular parasite *T. gondii*. The HFF cells were maintained in Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% cosmic calf serum, 5 μ g/mL streptomycin, and 5 units/ml penicillin in a humidified atmosphere containing 5% CO₂ at 37°C. The culture media was replaced with fresh

media every 4-5 days. The cells were grown as a monolayer on either tissue culture flasks or dishes.

Once the cells became confluent, the old medium was aspirated and the cells were detached by incubating with 1mL of a 0.25% trypsin-0.03% EDTA solution about 2 min at 37°C. Then the fresh complete media was added and the cells were resuspended and split into 3-4 times area of the culture flasks or culture dishes.

To freeze cells for later use, cell pellets were resuspended in cell freezing medium containing 12.5% dimethyl sulfoxide (DMSO) and 10% FBS in MEM and were immediately placed in freezer vials and stored at –80°C until further use.

3.3.2 *T. gondii* culture and manipulation

The *T. gondii* wild type (RH) strain was obtained from NIH, Division of AIDS research and cultured in minimal essential medium (MEM) supplemented with 1% dialyzed fetal bovine serum and 5 µg/mL gentamicin, and 5 units/mL penicillin, 5 µg/mL streptomycin (completed Ed1 Medium). After infecting a monolayer of confluent HFF cells 3-5 days, the lysed parasites were scraped and passed through a 27-G½ syringe needle and subsequent filtered through a 3 µm polycarbonate filter to remove the host cell debris. The parasites were centrifuged at 2,000 rpm for 10 minutes at room temperature to get the pellet and the pellet was washed twice with Dulbecco's phosphate-buffered saline with 0.1 g/L CaCl₂ (DPBS) before stored in –80°C freezer for future genomic DNA or RNA extraction.

To freeze the parasites, pellets without washing were resuspended in parasite freezing medium containing 12.5% DMSO and 10% dialyzed fetal bovine serum (dFBS) in MEM and then stored at -80°C .

3.3.3 Uracil incorporation assay

Extracellular *T. gondii* cells lysed from confluent HFF monolayers in one cell culture dish were pelleted, washed twice with DPBS, and resuspended in 800 μL electroporation buffer (cytomix buffer: 120 mM KCl, 0.15 mM CaCl_2 , 10 mM $\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$, pH 7.6, 25 mM HEPES, pH 7.6, 2 mM EDTA, 5 mM MgCl_2) that was freshly supplemented with 2 mM ATP and 5 mM glutathione. Various amounts of UPRz1, UPRz2, UPRz3, UPRz4 RNA, yeast tRNA, and wild type trans-acting delta ribozyme were added to electroporation buffer with parasites. Electroporations were performed in 4 mm electroporation cuvettes at 1.5K EV pulse R = 24 (pulse = 0.25 msec) using BTX model 630 Electro Cell Manipulator. After incubating for 15 minutes at room temperature, the contents of the electroporation cuvettes were inoculated into confluent monolayers of HFF cells grown in 24-well plates (about 10^4 parasites per well). Unattached parasites were removed by replacing the new medium after 4 hours incubation at 37°C . ^3H -uracil (2 μCi) was added to the 24 hours culture and then incubated for another 2 hours. The tray was chilled at -20°C for 2 min and one milliliter of ice-cold 0.6 N trichloroacetic acid (TCA) was added to the existing medium. The monolayer was fixed by incubating on ice for 1 hour. Unincorporated uracil was removed and rinsed in running water bath at least 4 hours. After the plate was dried in the air for few minutes, 0.5 mL 0.1 N NaOH was then added to each well and incubated at 37°C for

1 hour to dissolve the TCA precipitate. The amount of incorporated ^3H -uracil was measured using a scintillation counter by measuring the radioactivity remaining in the 0.5 mL TCA precipitate using 2.5 mL scintillation fluid. Each sample was tested at least three times.

3.3.4 Hypoxanthine uptake assay

HXRz1, HXRz2, yeast tRNA, and wild type trans-acting delta ribozyme (1 μg) were electroporated into freshly lysed *T. gondii* as described above. Confluent monolayers of HFF cells grown in 24-well plates were infected with transformed *T. gondii* parasites (about 10^4 parasites per well). After 4 hours incubation at 37°C , unattached parasites were removed by replacing the new medium. ^3H - hypoxanthine (2 μCi) was added to each of the 24 hours culture and then incubated for another 2 hours. The tray was chilled at -20°C for 2 min followed by addition of 1 mL ice-cold 0.6 N trichloroacetic acid (TCA) and incubation on ice for 1 hour. Unincorporated ^3H -hypoxanthine was removed and rinsed in running water bath at least 4 hours. NaOH (0.1 M) was then added to the dried each well and incubated at 37°C for 1 hour. The amount of incorporated ^3H -hypoxanthine was measured using a scintillation counter by measuring the radioactivity remaining in the 0.5 mL TCA precipitate using 2.5 mL scintillation fluid. Each sample was tested at least three times.

3.3.5 Northern-blot

3.3.5.1 Synthesis of DIG-labeled probe

UPRT and *HXGPRT* probes were made by PCR by using T7on5'UP and 3'UPRT

as primers and pUC18 T7GGG as template for *UPRT* probe, while using T7on5'HX and 3'HXGPRT as primers and pTu88mycHisGFPHX as template for *HXGPRT* probe. Digoxigenin-II-dUTP (DIG-dUTP) (0.5 nmol) was added to the PCR mixture and the mixture was subjected to the same PCR condition described before.

3.3.5.2 RNA extraction from *T. gondii*

After electroporation with 1 µg of UPRz2, HXRz2 and their mixture respectively, parasites were incubated at 37°C CO₂ incubator for 24 hours. RNA was then extracted from the parasite pellets using Trizol reagent according to the manufacturer's instructions. Briefly, Trizol Reagent (500 µL) was added to the parasite pellet prepared as described before. After adding 100 µL of chloroform, the sample was shaken vigorously for 15 seconds and incubated at room temperature for 2 min. The phase was separated after centrifugation at 12,000 rpm at 4°C. RNA was precipitated by isopropyl alcohol, washed by 75% ethanol and dissolved back in 30 µL of DEPC-treated water. RNA concentration was quantified by UV spectrophotometer. The extracted RNA was treated with 2 units of RQ1 RNase-free DNase for 15 min at 37°C, and the DNase was inactivated by heating at 75 °C for 5 minutes.

3.3.5.3 Northern blot analysis

About 10 µg of total RNA in 2 volumes RNA loading buffer (50% formamide, 5.5% RNA-grade formaldehyde, 0.25% of each Bromophenol Blue and Xylene Cyanol, 20 mmol/L MOPS, 5 mmol/L sodium acetate, and 1 mmol/L EDTA pH 7.0) was denatured at 70°C for 15 min and chilled on ice before being loaded on 1% agarose /2.7%

formaldehyde gel run in MOPS buffer (20 mmol/L MOPS, 5 mmol/L sodium acetate, and 1 mmol/L EDTA pH 7.0) at 50 volts. The RNA was transferred to Hybond-N⁺ membrane from the gel by either capillary blotting (Sambrook et al., 1989) or by Vacuum Blotter. UV was used to crosslink the RNA onto the membrane (500 mJ setting for 1 min). The membrane was prehybridized in prehybridization buffer (5xSSC, 0.05 M NaPB pH 7.0, 1% blocking solution, 0.1% sarcosyl, and 0.02% w/v SDS) in hybridization oven at 68°C for 4 hours and hybridized with 3 mL fresh prehybridization buffer containing 8 µL heat denatured DIG-labeled probe at 68°C for more than 16 hours. Then, the membrane was washed with 2X SSC, 0.1% SDS for 5 min at room temperature and washed twice with 0.5X SSC, 0.1% SDS for 30 min at 68°C. After being soaked in 20 mL maleic acid, the membrane was treated with 20 mL blocking solution for 30 min followed by 20 mL antibody solution (anti-DIG alkaline phosphatase Fab diluted 1 in 10,000) for 30 min. The signals were detected using the chemiluminescent substrate CSPD according to the manufacturer's instructions. Chemiluminescent signals were analyzed by using the chemiluminescence filter wheel in Alpha Imaging system.

3.4 Characterization of a stable *T. gondii* cell line expressing engineered delta ribozymes

3.4.1 Selection of stable cell line

The constructed plasmids, pUC19-UPRz2 (10-20 µg), were introduced into a number of 10⁷ *T. gondii* tachyzoites by electroporation as described above. The parasites were selected 24 hours after electroporation by replacing the new completed ED1 medium with the supplementary 5-fluoro-2'-deoxyuridine (FDUR) at 5 µM final concentration. The

parasites were passed for other three round of drug selection to select for stable cell line. The stable cell line parasites were either subcultured onto HFF in the presence of FDUR or stored at -80°C for further studies.

3.4.2 Polymerase Chain Reaction (PCR) and Southern blot

3.4.2.1 Genomic DNA extraction from *T. gondii*

The pellets of both transformed and wild type *T. gondii* culture were suspended in 450 μL lysis buffer consisting of 100 mM EDTA, 10 mM Tris pH 8.0, 1% SDS, and 2 mg/mL proteinase K. After an overnight incubation at 43°C , the suspension was extracted with an equal volume of Tris EDTA buffered phenol: chloroform: isoamyl (25: 24: 1) and followed by equal volume of chloroform. The upper aqueous layer containing the nucleic acid was removed into a new tube. One tenth volume of 3 mol/L sodium acetate (pH 5.3) and two volumes of 95% ethanol were added to the aqueous phase to precipitate the genomic DNA. After incubation at -20°C for 15 min, the sample was centrifuged at 12,000 rpm for 15 min at 4°C . The precipitated DNA was washed with 75% ethanol and air dried in an inverted position for about 15 min. The pellet was resuspended in 30 μL of TE buffer and then stored at -20°C for further use.

3.4.2.2 PCR

PCR was carried out in a total volume of 50 μL by using about 100 ng of genomic DNA of stable cell line parasites as template. Universal primer and reverse (Appendix Table) which covered the ribozyme sequence region were used as forward and reverse primers. PCR was run for 30 cycles of denaturation (95°C , 30 sec), annealing (55°C , 30

sec) and extension (72°C, 45 sec). PCR products were checked on 1% agarose gel.

3.4.2.3 Southern blotting

The probe was generated by PCR using p3100RZ plasmid template and universal and reverse primers under the same condition described before. The resulting PCR product (750 nt) was labeled with digoxigenin-dUTP (DIG-dUTP).

Genomic DNA (10 µg) isolated from both stable cell line and wild type parasites were digested with *BamH*I and fractionated by electrophoresis through 0.7% agarose gel at 30 volts. The gel was then denatured in the denaturing solution (0.5 M NaOH, 1.5 M NaCl) for 30 min at room temperature, and neutralized in neutralizing solution (0.5 M Tris-pH7.2, 1.5 M NaCl, 1 mM EDTA) twice for 15 min. The DNA was transferred from the gel to Hybond-N+ nylon membrane and the hybridization and detection were performed as described in Northern blot.

3.4.3 RT-PCR

Total RNA from the pellets of both stable transformants and wild type *T. gondii* was extracted using Trizol reagent. One microgram of DNase treated total RNA was used as initial template for the RT reactions. Twenty microliter reactions containing 10 pmoles of ribozyme RT-Primer, template and sterile DEPC-H₂O were heated at 70°C for 5 min and chilled on ice for 2 min. Tris-HCl (50 mM, pH 8.3), 75 mM KCl, 10 mM DTT, 3 mM MgCl₂, 0.5 mM of each dNTP, 50 units of RNase out RNase inhibitor and 200 units of M-MLV reverse transcriptase were added and incubated at 37°C for 60 mins. The reaction was inactivated by heating at 95°C for 1 min. Eight microliter of cDNA from the

RT reaction mixtures were used as the template for the subsequent PCR amplification. Each HxoI-RZ-FW and ribozyme RT-Primer (50 pmoles) were used as forward and reverse primers. The PCR was performed in a total volume of 100 μ L containing 75 mM Tris-HCl (pH 8.8), 50 mM KCl, 2 mM MgCl₂, 50 μ M dNTPs, and 2 units of Taq DNA polymerase. The reactions were programmed for 30 amplification cycles of denaturation at 94°C for 30 sec; annealing at 56°C for 30 sec and extension at 72°C for 1 min. The products were fractionated by 10% polyacrylamide gel and then stained in 5 ng/mL of ethidium bromide TBE buffer for about 15 min, and checked with the AlphaImager 2200 Light Imaging System.

CHAPTER 4

RESULTS

The obtained results are described in three sections. The first section deals with data related to the engineering of delta ribozymes and their *in vitro* cleavage activities. The second section reports results related to the effects of engineered ribozymes on the level of *UPRT* and *HXGPRT* gene expression in *T. gondii*. The final part is about the characterization of a stable cell line of *T. gondii* expressing the engineered delta ribozymes against *UPRT*.

4.1 Engineering of delta ribozymes and their *in vitro* cleavage activity

4.1.1 Selection of targets

To design delta ribozymes specific to *UPRT* and *HXGPRT* mRNA, the secondary structures of the target mRNAs were predicted by using the MFOLDTM program. The entire open reading frame of *UPRT* and *HXGPRT* cDNA sequences of *Toxoplasma gondii* are shown in Table 4.1 (A) and Table 4.2 (A) respectively. Figure 4.1 (A, B) shows the predicted secondary structures of *UPRT* and *HXGPRT* mRNA which are 725 nt and 603 nt long. Four sites within *UPRT* mRNA (at position of 38 nt, 171 nt, 404 nt, 544 nt) and two sites within *HXGPRT* mRNA (at position of 169 nt, 335 nt) were chosen for the design of different delta ribozymes. Target sequences are illustrated in Table 4.1 (B), Table 4.2 (B). The targeted sites were specifically selected because they form only single-stranded regions which would be more accessible to ribozymes than double-stranded regions.

FIGURE 4.1

Predicted Secondary Structure of *UPRT* and *HXGPRT* mRNA by MFOLD™

Program

Legend

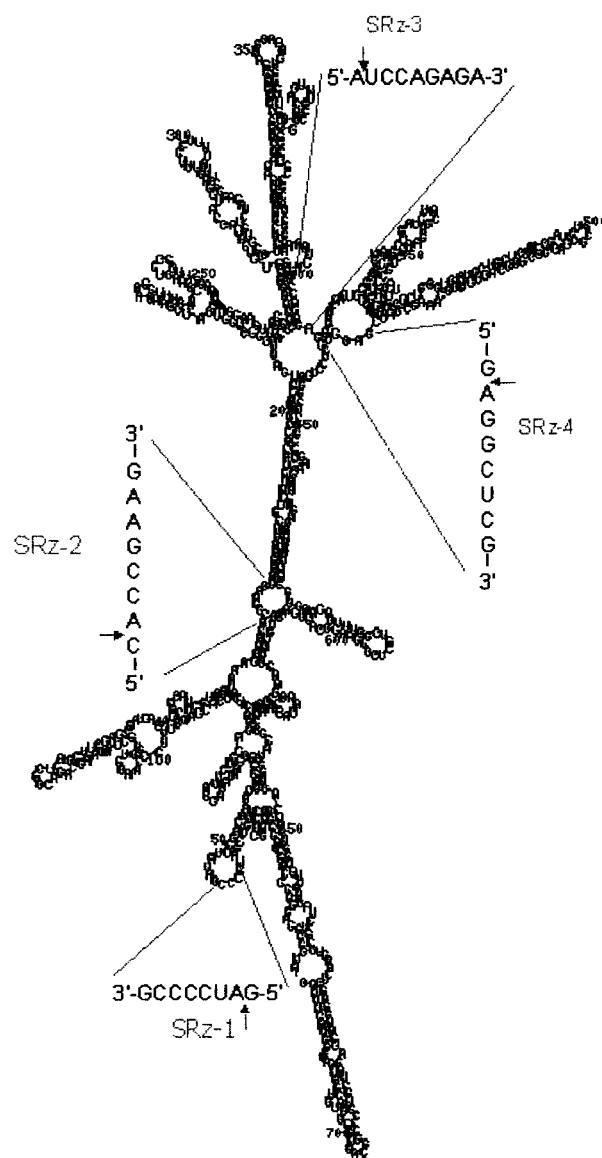
(A) Predicted secondary structure of *UPRT* 725 nt mRNA.

(B) Predicted secondary structure of *HXGPRT* 603 nt mRNA

Chosen targets and their sequences are shown. The arrows indicate the cleavage sites.

FIGURE 4.1 A

plt22.jpg by D. Stewart and M. Zuker
 © 2003 Washington University



d6 = -222.38 [initially -246.21 03May01-17-07-05

Figure 4.1 B

prepared by Dr. Steven A. Zele
 at the Washington University

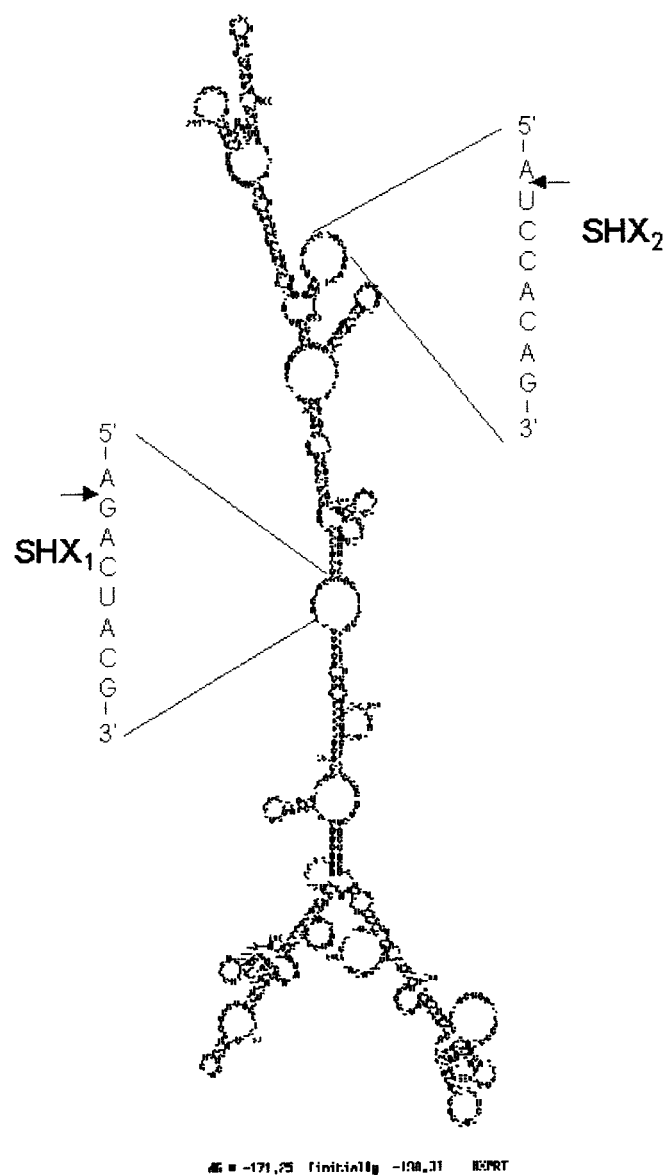


Table 4.1A *UPRT* cDNA Sequence

ATGGCGCAGG	TCCCAGCGAG	CGGAAAGCTC	CTTGTCGATC	CCCGATATTC	GACAAACGAC
CAGGAAGAAA	GCATTCTCCA	GGACATCATC	ACGAGGTTC	CCAATGTGGT	GCTCATGAAG
CAGACGGCTC	AGCTTCGAGC	GATGATGACC	ATCATTCGTG	ATAAAGAAAC	ACCGAAGGAA
GAATTCGTCT	TCTACGCCGA	CCGCCTGATT	CGCCTCCTCA	TCGAAGAAGC	TTTGAACGAA
CTGCCGTTTC	AAAAGAAGGA	AGTGACAACC	CCTCTGGATG	TGTCATACCA	TGGAGTTTCC
TTTTATTCCA	AGATCTGTGG	CGTCTCGATT	GTGAGAGCTG	GCGAGTCGAT	GGAAAGTGGC
TTGCGGGCAG	TTTGCCGCGG	CTGCCGCATC	GGGAAAATCC	TCAT TCCAGAG	AGACGAAACA
ACTGCGGAGC	CTAAGCTGAT	CTACGAGAAG	CTGCCTGCCG	ACATTAGAGA	TCGCTGGGTG
ATGCTGCTCG	ATCCGATGTG	CGCGACGGCG	GGCAGTGTGT	GCAAAGCGAT	CGAGGTCCTC
CTG AGGCTCG	GCGTGAAGGA	AGAGAGAATC	ATTTTCGTCA	ACATTTTGGC	TGCTCCCCAA
GGCATTGAAC	GTGTTTTCAA	GGAATACCCG	AAAGTCCGCA	TGGTCACTGC	TGCTGTTGAC
ATCTGCCTGA	ACTCGAGGTA	CTACATCGTC	CCCGGCATTG	GTGATTTTCG	TGACCGGTAC
TTTGGAACCA	TGTAG				

Table 4.1B Cleavage Sites of UPRz Ribozymes on *UPRT* mRNA

Ribozyme	Target <i>UPRT</i> cDNA Sequence	Sites	Product
UPRz1	5'-AUCCCCG-3'	38 nt	37 nt and 681 nt
UPRz2	5'-ACCGAAG-3	171 nt	170 nt and 558 nt
UPRz3	5'-UCCAGAG-3'	404 nt	403 nt and 325 nt
UPRz4	5'-AGGCUCG-3'	544 nt	543 nt and 185 nt

Table 4.2A *HXGPRT* cDNA Sequence

ATGGCGTCCA	AACCCATTGA	AGAATCCCGG	TCGCAAAAAC	GGAGTGCCTT	CTCAGACATC
TTCTGTTGTT	GCACTCCTAA	TGAAGGGGCT	ATCGTGCCCA	GTGACCCAAT	GGTCTCCACC
AGTGCTCCAG	CACGCACCAG	TGCTCCAGCG	CGCTCCAGTG	CACTTCAAGA	CTACGGCAAG
GGCAAGGGCC	GTATTGAGCC	CATGTATATC	CCCGACAACA	CCTTCTACAA	CGCTGATGAC
TTTCTTGTGC	CCCCCCTG	CAAGCCCTAC	ATTGACAAAA	TCCTCCTCCC	TGGTGGATTG
GTCAAGGACA	GAGTTGAGAA	GTTGGCGTAT	GACAT CCACA	GA ACTTACTT	CGGCGAGGAG
TTGCACATCA	TTTGCATCCT	GAAAGGCTCT	CGCGGCTTCT	TCAACCTTCT	GATCGACTAC
CTTGCCACCA	TACAGAAGTA	CAGTGGTCGT	GAGTCCAGCG	TGCCCCCCTT	CTTCGAGCAC
TATGTCCGCC	TGAAGTCCTA	CCAGAACGAC	AACAGCACAG	GCCAGCTCAC	CGTCTTGAGC
GACGACTTGT	CAATCTTTCG	CGACAAGCAC	GTTCTGATTG	TTGAGGACAT	CGTCGACACC
GGT					

Table 4.2B Cleavage Sites of HXRz Ribozymes on *HXGPRT* mRNA

Ribozyme	Target <i>HXGPRT</i> cDNA Sequence	Sites	Product
HXRz1	5'-GACUACG-3'	169 nt	168 nt and 435 nt
HXRz2	5'-UCCACAG-3	335 nt	334 nt and 269 nt

4.1.2. Sequencing analysis of the constructed plasmids

The DNA sequences of the constructed plasmids were determined by the Sanger dideoxynucleotide sequencing method. Figure 4.2A shows the sequence of a segment of the *trans*-acting delta ribozyme encompassing nucleotides 20 to 27, which corresponds to P1 stem (Fig. 1.2). The X region is the 7 nt P1 stem that was replaced by the corresponding recognition sequence for each designed delta ribozyme. The *Sph*I and *Rsr*II restriction sites are indicated with arrows. Sequencing analysis (Fig. 4.2 B-G) confirmed that the X region within the P1 stem of each of the plasmids pUC19-UPRz1, pUC19-UPRz2, pUC19-UPRz3, and pUC19-UPRz4 (Fig. 4.2 B-E) was replaced by the exactly complimentary sequences of the corresponding chosen target sites within *UPRT* mRNA. For plasmid pUC19-UPRz1, for instance, which was designed based on the selected target sequence 5'-AUCCCCG-3', the determined sequence of the X region within the P1 stem was 5'-CGGGGAU-3'. Similarly, HXRz1 and HXRz2 P1 stem sequences (Fig. 4.2, F, G) were modified to specifically target *HXGPRT* mRNA substrate sites.

FIGURE 4.2

Sequence Confirmation of the Constructed Plasmids

Legend

(A) Sequence of a segment of the ribozyme encompassing nucleotides 20 to 27 corresponding to P1 stem of the *trans*-acting delta ribozyme (Fig. 1.2). X region is the 7 nt P1 stem that was changed for the specific targets. Arrows indicate the *Sph*I and *Rsr*II restriction sites.

(B)-(G) Autoradiograms of the sequencing gels. The constructed plasmids, named pUC19-UPRz1, pUC19-UPRz2, pUC19-UPRz3, pUC19-UPRz4, pUC19-HXRz1, pUC19-HXRz2 have the sequence of the P1 stem modified. The modified sequences are showed along the gels.

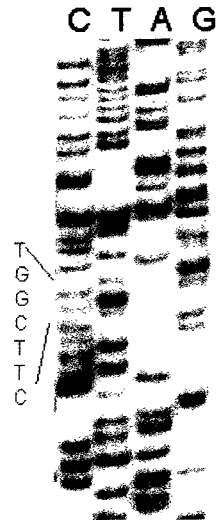
Figure 4. 2

A.

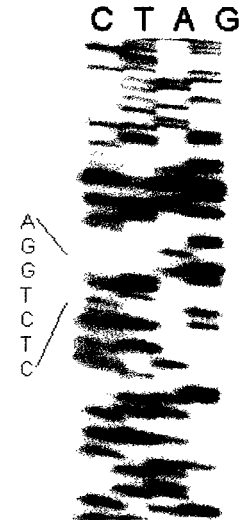
GG GTC CAC CTC CTC GCG[▼]GTX XXX XXX GGG
CAT GCG GCT TCG CAT GGC TAA GGG ACC C



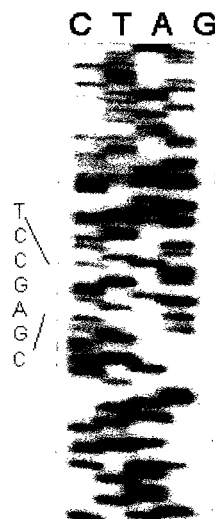
B. pUC19-UPRz1



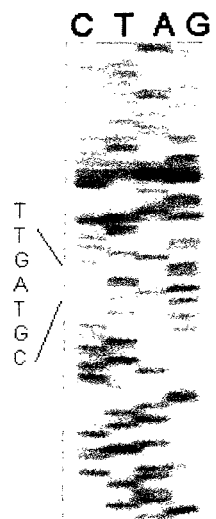
C. pUC19-UPRz2



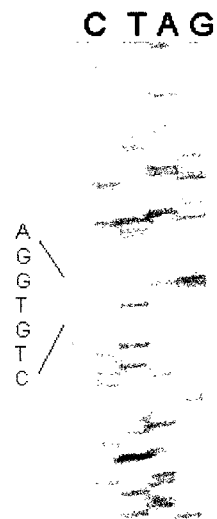
D. pUC19-UPRz3



E. pUC19-UPRz4



F. pUC19-HXRz1



G. pUC19-HXRz2

4.1.3. Cleavage activity of engineered delta ribozymes using model substrates (15 nt)

In order to determine the cleavage activity of the engineered ribozymes without any interference from the folding of RNA substrates, *in vitro* cleavage experiments were carried out using 15 nt long substrates (12 nt fragment of *UPRT* target site sequence ending with GGG). Each constructed ribozyme and its complementary substrate (15 nt) were incubated at 37° C for 1 hour under the same conditions. No cleavage products were detected when UPRz1, UPRz3, and UPRz4 were used. Only UPRz2 could cleave its substrate. To further test the specificity of UPRz2 cleavage activity, additional cleavage reactions were carried out using the three other different substrates for UPRz1, UPRz3, and UPRz4. Data shows that UPRz2 could cleave its own complimentary substrate only and not the other three non-complimentary substrates (Fig. 4.3).

A time course cleavage experiment was carried out using different concentrations of UPRz2 (5 nM - 600 nM) and a trace amount (2.5 nM) of its model substrate. The data is shown in Fig. 4.4. The percentage of cleaved substrate was calculated following the quantification of the cleavage products and the substrates by Optiquan programTM that based on densitometric analysis. As shown in Fig. 4.5A, with 600 nM UPRz2, the maximum percentage of cleaved substrate was 17.99% at 120 min. The data demonstrated that the amount of cleavage products (6 nt long) increased with the reaction time, and that to some extent, higher amounts of the ribozyme could cleave its substrate in less time. The observed rate constant calculated using software

GRAFITTM was $0.014 \pm 0.002 \text{ min}^{-1}$. Figure 4.5 B shows the dependence of the ribozyme cleavage activity on ribozyme concentration.

FIGURE 4.3

Cleavage Activity and Specificity of UPRz2 Using Model Substrates (15 nt)

Legend

Autoradiogram of a 20% denaturing polyacrimide gel revealing the cleavage activity and specificity of UPRz2. UPRz2 can only cleave its own complimentary substrate and produce product (6 nt), not the substrates for UPRz1, UPRz3, and UPRz4 in one hour reaction time at 37° C.

Figure 4. 3

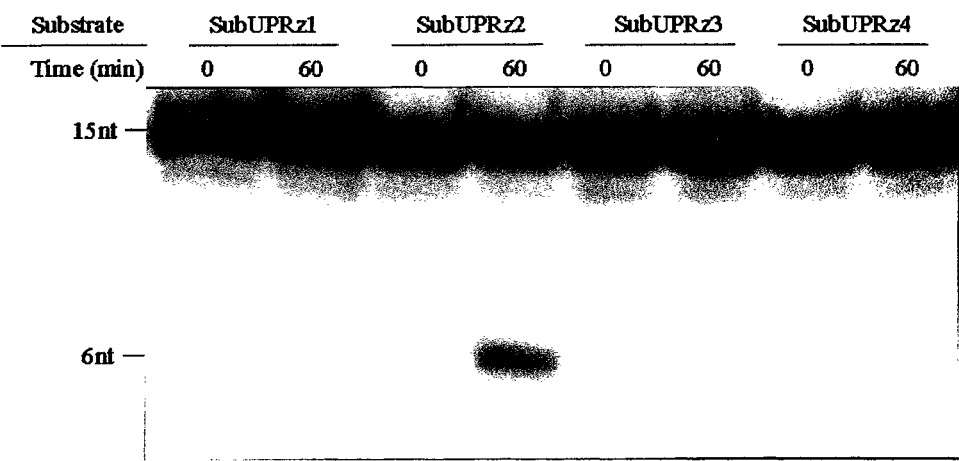
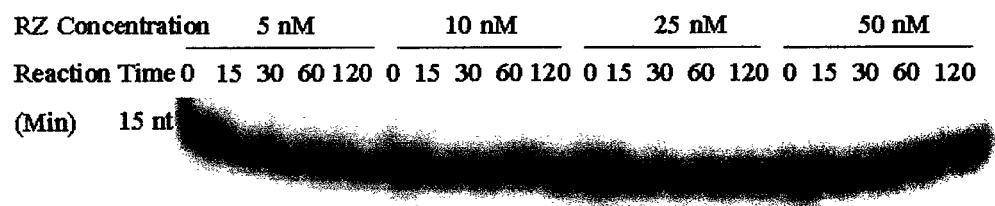


FIGURE 4.4

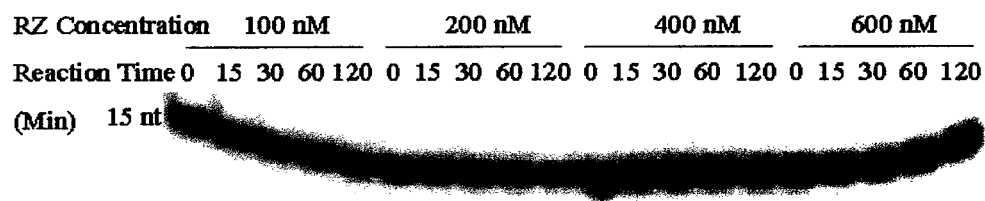
Ribozyme Cleavage Assay Using Model Substrates (15 nt)

Autoradiogram of a 20% denaturing polyacrimide gel for the analysis of the cleavage activity of UPRz2. Final concentration as 5 nM, 10 nM, 25 nM, 50 nM, 100 nM, 200 nM, 400 nM, 600 nM of UPRz2 individually incubated with trace amount (2.5 nM) of its *UPRT* model substrate in 0 min, 15 min, 30 min, 60 min, 120 min reaction time points. The amount of cleavage products (6 nt) increased with the reaction time and, to some extent, with the concentrations of ribozyme.

Figure 4. 4



6 nt



6 nt

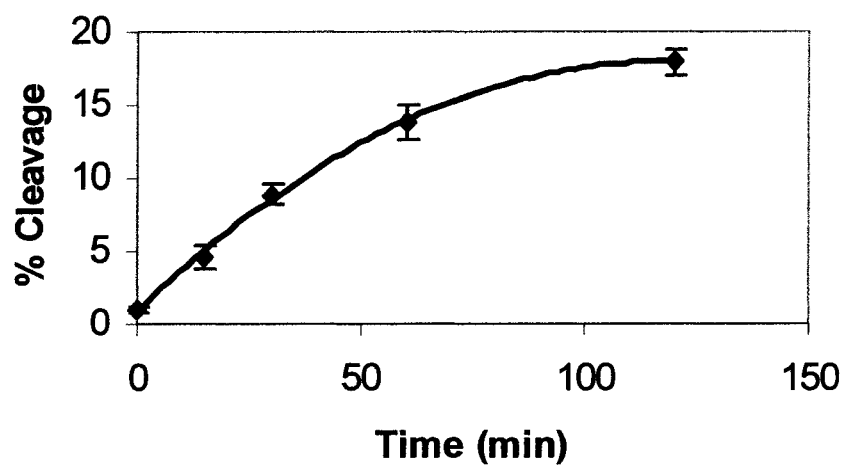
FIGURE 4.5

Kinetic Analyses of UPRz2

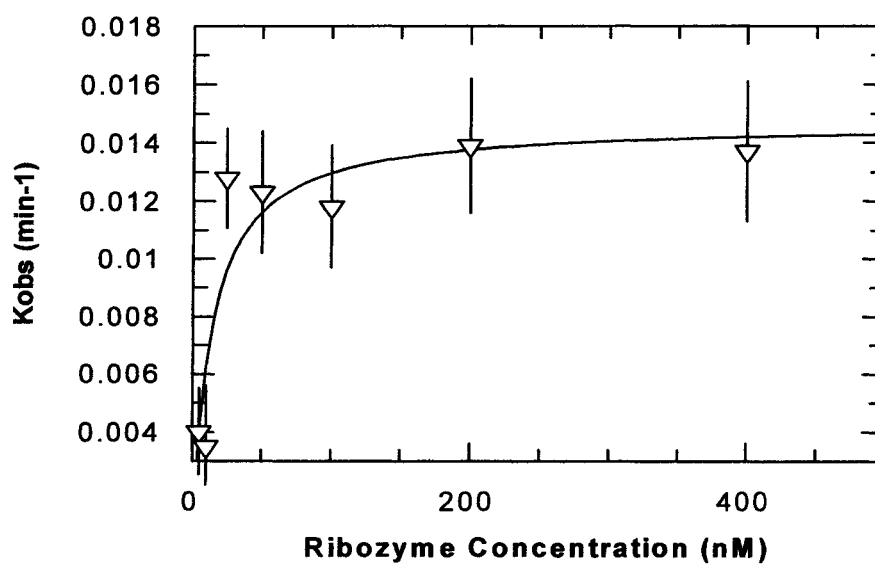
Legend

- A. Time course analysis of ribozyme cleavage activity (UPRz2 concentration was 600 nM)
- B. Dependence of the ribozyme cleavage activity on ribozyme concentration

Figure 4. 5



$$K_{obs} = 0.014 \pm 0.002 \text{ (min}^{-1}\text{)}$$



4.1.4 The effect of *T. gondii* cell-free extract on ribozyme activity

To test the effect of protein of RH parasites on the activity of UPRz2, cleavage assays were carried out in the presence of RH *T. gondii* cell-free extract. Cell lysates with 0.3 µg/µL total protein concentration were used. Cleavage products (6 nt) could be detected in one hour for those reactions carried out with cell lysate of final protein amount ranging from 0 to 1.5 µg. However, when the amount of RH parasite lysate was increased to 10 µL in the reaction (3 µg of protein), UPRz2 could not cleave its substrate as evidenced by the absence of cleavage products (Fig. 4.6A). Also the substrate RNA showed random degradation.

To further test the effect of protein of RH parasites on kinetic of UPRz2, different concentrations of UPRz2 (5 nM – 600 nM) were incubated with a trace amount (2.5 nM) of end-labeled substrate in the presence of 2 µL parasite lysate (0.6 µg of protein). The reactions were aliquoted at different time intervals and finally run on polyacrylamide gel. Figure 4.7 shows that the amount of cleavage products (6 nt) increased with the reaction time and ribozyme amount, as was previously shown for cleavage reactions conducted without the parasite lysate (Fig. 4.4). This experiment demonstrated that UPRz2 could function in the presence of *T. gondii* cellular proteins. The observed rate of cleavage reaction with cell-free extract was $0.047 \pm 0.031 \text{ min}^{-1}$ when UPRz2 concentration was 600 nM, suggesting that *T. gondii* proteins might have some effects on the ribozyme activity.

The lysis buffer used to break the *T. gondii* cell contained 1% SDS (v/v) and 0.2 mol/L NaOH. To eliminate the effect of the lysis buffer on the cleavage activity, 200

nM UPRz2 was incubated with trace amount of short substrate in lysis buffer only under the same condition. Fig. 4.6B showed that UPRz2 functioned and the cleavage product increased with the reaction time.

FIGURE 4.6

The Effect of *T. gondii* Cell-free Extract on Ribozyme Activity

Legend

A. Autoradiogram of a 20% denaturing polyacrylamide gel for the effect of *T. gondii* cell-free extract on the cleavage activity of UPRz2 in one hour reaction time at 37° C.

Lane 1. 250 nM UPRz2 + 2.5 nM substrate + RH extract with 0.6 µg protein

Lane 2. 2.5 nM substrate + RH extract with 0.6 µg protein only

Lane 3. 250 nM UPRz2 + 2.5 nM substrate + RH extract with 3 µg protein

Lane 4. 2.5 nM substrate + RH extract with 3 µg protein only

B. Autoradiogram of a 20% denaturing polyacrylamide gel for the analysis of the effect of lysis buffer on UPRz2 Activity

Lane 1-5 are the samples taken from reaction time 0, 15, 30, 60, 120 min respectively.

Figure 4. 6

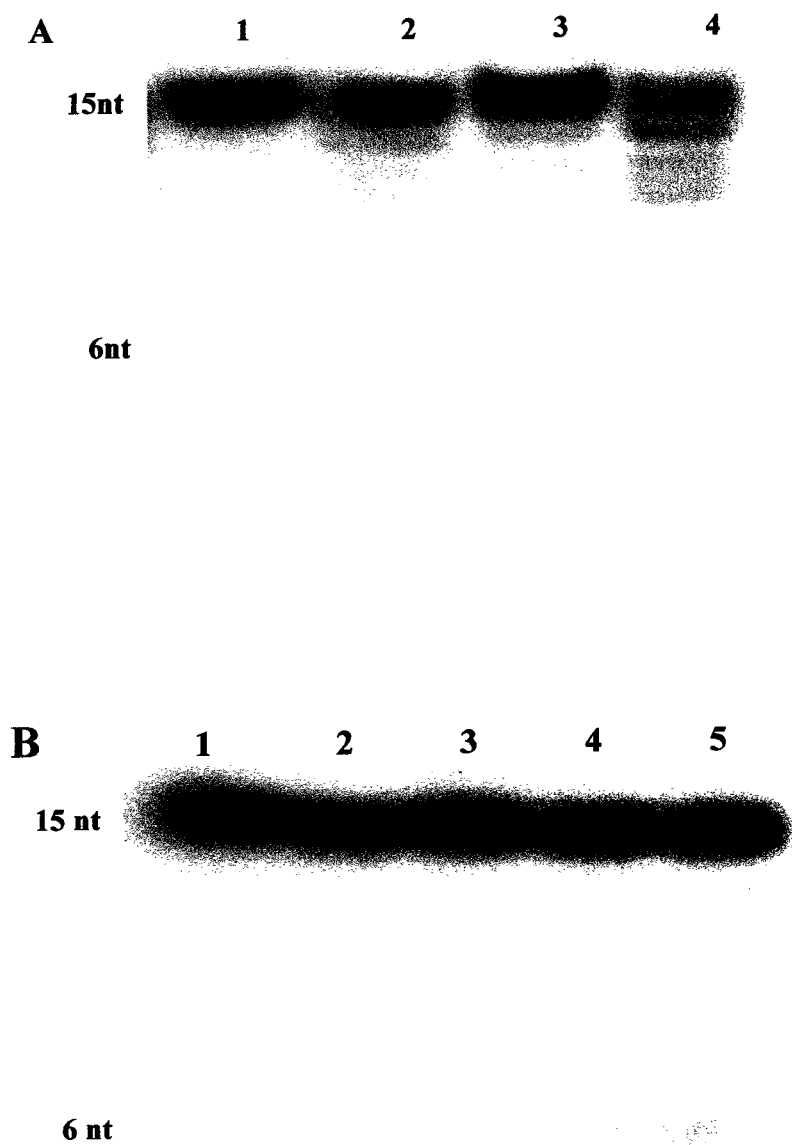


Figure 4.7


Ribozyme Cleavage Assay Using Model Substrates with Presence of RH Cell-free

Extract


Legend

Autoradiogram of a 20% denaturing polyacrylamide gel for the analysis of the cleavage activity of UPRz2 in presence of RH cell-free extract. Two microliter of RH cell-free extract (with 0.6 µg protein) was added to the reaction of different concentration (5 nM, 10 nM, 25 nM, 50 nM, 100 nM, 200 nM, 400 nM, 600 nM) of UPRz2 with trace amount (2.5 nM) of its short *UPRT* target substrate in 0 min, 15 min, 30 min, 60 min, 120 min reaction time points.

Figure 4.7

Ribozyme Concentration		5 nM					10 nM					25 nM					50 nM				
Cleavage Reaction Time		0	15	30	60	120	0	15	30	60	120	0	15	30	60	120	0	15	30	60	120
(Min)	15 nt																				

6 nt

Ribozyme Concentration		100 nM					200 nM					400 nM					600 nM				
Cleavage Reaction Time		0	15	30	60	120	0	15	30	60	120	0	15	30	60	120	0	15	30	60	120
(Min)	15 nt																				

6 nt

4.1.5 Cleavage activity of ribozymes using long *UPRT* substrates

To verify whether the engineered ribozymes could cleave the long *UPRT* mRNA, we synthesized longer substrates (150-720 nt) which are corresponding to *UPRT* mRNA and incubated them with engineered delta ribozymes under the same conditions as with model substrates. However, there was no specific cleavage products had been detected. Primer extension, or run-off reverse transcription reactions were carried out to further test the cleavage activity of engineered delta ribozymes on long *UPRT* mRNA substrates at the expected sites (strategy illustrated in Fig. 4.8A). Different sizes of *UPRT* DNA fragments were produced by PCR using T7 promoter region oligonucleotide as a forward primer and a designed oligonucleotide (50-80 nts downstream from the cleavage sites) as a reverse primer. *UPRT* mRNA substrates were synthesized by *in vitro* transcription using PCR products as templates. The synthesized substrates were then subjected to cleavage by the engineered ribozymes under the optimized conditions. The resulting products were reverse transcribed into ^{32}P labeled cDNA which would run-off at the end of the transcripts. Figure 4.8B demonstrated that the cDNA product was detected in the reaction of UPRz2 (750 nM) with labeled synthesized substrate and the RT product was run-off at the expected cleavage site. Control reactions that is without UPRz2 did not show any product.

Similarly, primer extension reactions were done for UPRz1, UPRz3 and UPRz4. By increasing the amount of UPRz4 from 750 nM to 1.5 pM, the cDNA product was detected at the corresponding cleavage site. UPRz3 (750 nM) could also cleave its

substrate at the correct site, while for UPRz1 we could not detect any signal. The cDNA products for UPRz3 and UPRz4, however, gave weaker signals when compared to the product of UPRz2.

FIGURE 4.8

Confirmation of Engineered Delta Ribozyme Cleavage Site

Legend

- A. Schematic diagram describing the strategy for the Primer extension assay
- B. Autoradiogram of a sequencing gel revealing the cleavage site. Lane 1 is the reaction products for UPRz2 with *UPRT* mRNA substrate, and lane 2 is the reaction products for *UPRT* mRNA substrate only. The sequencing reaction and the corresponding sequences are shown. The arrow indicates the ribozyme cleavage site (within the RNA substrate).

C. Figure 4.8 A

A. Primer extension assay strategy

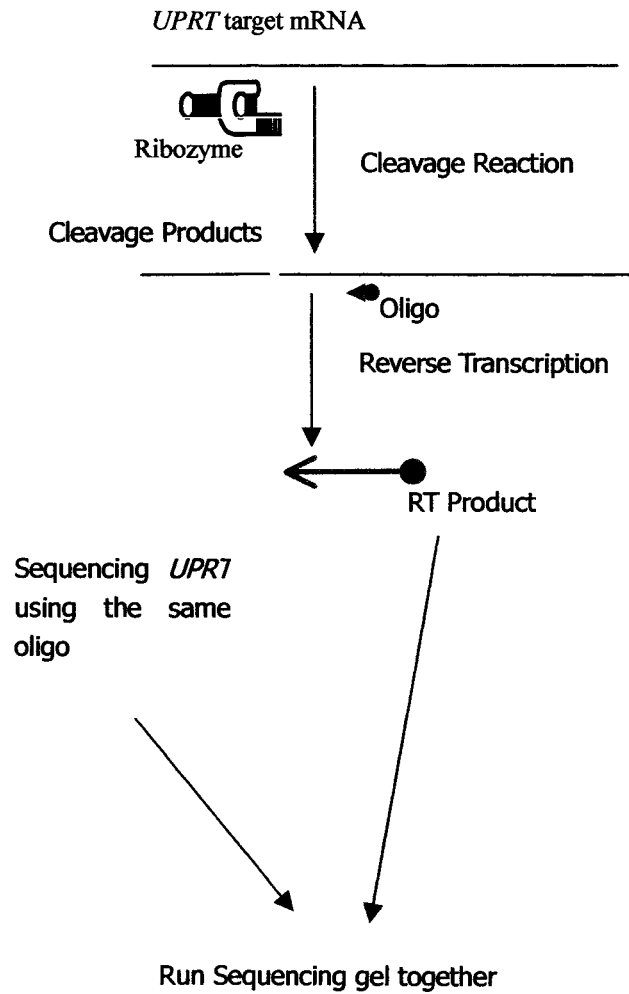
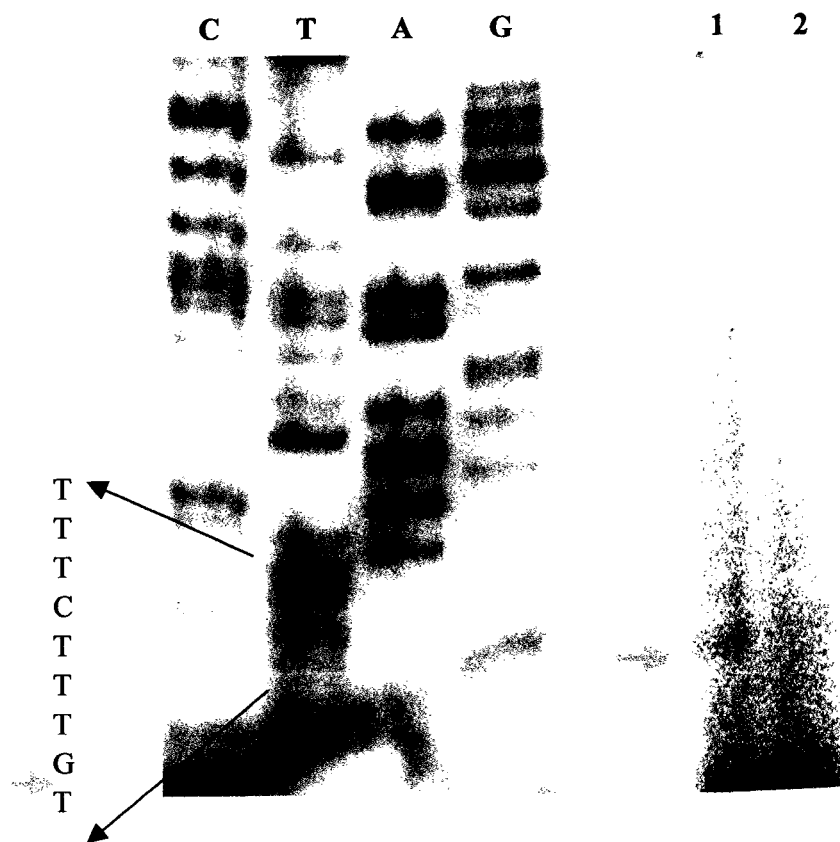


Figure 4.8 B



4.2 Effects of engineered ribozymes on the level of *UPRT* and *HXGPRT* gene expression in *T. gondii*

The cleavage activity of delta ribozyme could lead to the inhibition of *UPRT* and *HXGPRT* gene expression in *T. gondii* by lowering the level of *UPRT* and *HXGPRT* mRNA. This decrease in mRNA level would consequently lead to less *UPRT* and *HXGPRT* activity. Thus, the down-regulation effect of engineered delta ribozymes in *T. gondii* could be indicated by measuring *UPRT* and *HXGPRT* activity.

4.2.1 Effects of engineered ribozymes on the level of *UPRT* gene expression

Uracil incorporation assay was used to measure *UPRT* activity in the parasites electroporated with engineered *UPRz1*, *UPRz2*, *UPRz3*, and *UPRz4* RNA. Equal amounts (1 µg) of all tested RNAs were added into the electroporation buffer. A mock electroporation (with buffer instead of RNA) was always performed for each electroporation experiment. The relative values of *UPRT* activity were calculated as the ratio to the mock electroporation. To further control for nonspecific effects of RNA electroporation, we used two additional RNAs: yeast tRNA and wild type *trans*-acting delta ribozyme. As shown in Fig. 4.9, the *UPRT* activity was inhibited when *UPRz2*, *UPRz3*, and *UPRz4* RNA were transformed into the parasites, while the control RNAs, yeast tRNA and wild type *trans*-acting delta ribozyme, did not have any significant effect on the uracil uptake. *UPRz1* could only slightly lower the parasite's ability to uptake uracil ($48.6 \pm 14.8\%$), *UPRz2* lowered the parasite's ability to uptake uracil ($10.6 \pm 6.2\%$), while *UPRz3* and *UPRz4* lowered the uracil

assimilation to $14.4 \pm 8.8\%$ and $24.9 \pm 3.4\%$ respectively. The activity of UPRT was not significantly lowered by UPRz1 even though higher amounts (up to 5 μg) were used in the electroporation (Fig. 4.10A). However, it was observed that decreasing the amount of UPRz2, UPRz3, and UPRz4 as low as 0.01 μg resulted in less inhibitory effect on ^3H -uracil incorporation by the parasite (Fig. 4.10B, C, and D). A dose response effect on the *UPRT* gene expression was detected when UPRz2, UPRz3, and UPRz4 were used individually (Fig. 4.10B, C, D).

The activities of UPRT were measured for *T. gondii* parasites electroporated with lower amount of UPRz1, UPRz2, UPRz3, and UPRz4 (0.1 μg and 0.01 μg respectively) supplemented with WT *trans*-acting delta ribozymes (to 1 μg). The results (Fig. 4.11A, B, C, D) showed that the supplemented WT *trans*-acting delta ribozymes did not enhance the inhibitory effect of the engineered delta ribozymes on ^3H -uracil incorporation by the parasites. This suggested that only the active delta ribozymes could function as expression modulator for the specifically targeted gene.

To test whether the engineered delta ribozyme could down regulate UPRT activity more when two active ribozymes function together, a mixture of equal amount of UPRz2 with UPRz3, UPRz2 with UPRz4, and UPRz3 with UPRz4 (0.5 μg of each ribozyme) was electroporated into RH parasites. Fig. 4.12 showed that mixture of UPRz2 with UPRz3 could lower the UPRT activity most ($46.9 \pm 31.4\%$), while UPRz2 with UPRz4, and UPRz3 with UPRz4 could lower the uracil assimilation to $63.9 \pm 19.4\%$, $58.6 \pm 3.76\%$ respectively. However, the effect of the mixture was not more than, or even as much as, that of the single ribozyme.

FIGURE 4.9

Effect of Engineered Ribozyme on UPRT Activity in *T. gondii*

Legend

Histogram illustrates the level of ^3H -uracil radioactivity in *T. gondii* electroporated with engineered ribozymes. One microgram of UPRz1, UPRz2, UPRz3, UPRz4, yeast tRNA and wild type trans-acting delta ribozyme were electroporated into the parasites. The UPRT activity was inhibited by the engineered ribozymes, while the control RNAs, yeast tRNA and wild type *trans*-acting delta ribozyme (WT), did not have any significant effect. At least three independent experiments were conducted.

Figure 4. 9

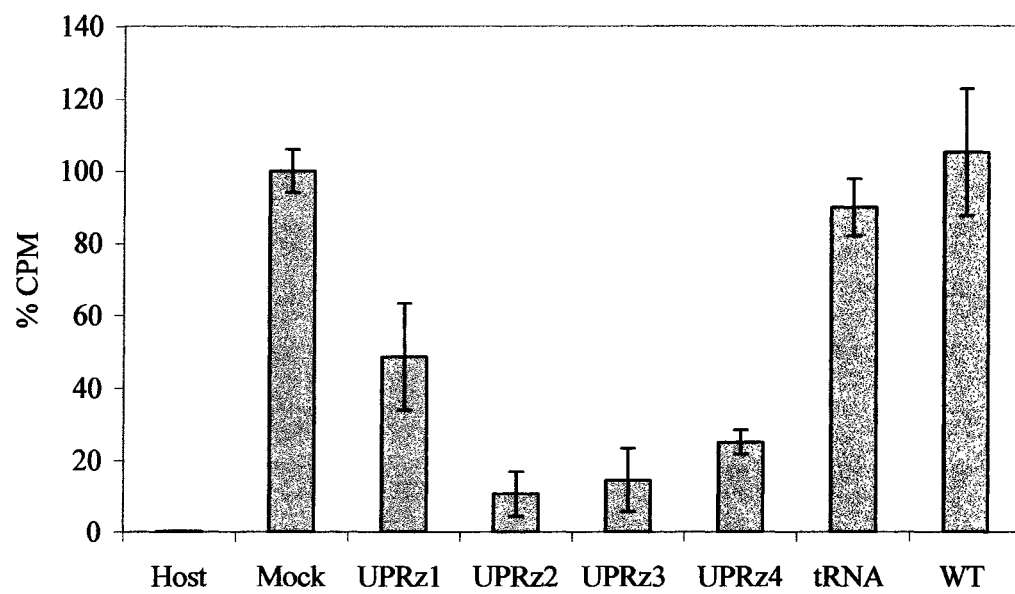


FIGURE 4.10

Dose dependent assays for the effect of engineered ribozymes on UPRT

activity in *T. gondii*

Legend

Histogram illustrates the level of ^3H -uracil radioactivity in *T. gondii* electroporated with the engineered ribozymes 1 μg , 2 μg and 5 μg of UPRz1(A). UPRz2 (1 μg , 0.1 μg and 0.01 μg) were electroporated (B). Same with UPRz3 (C), and UPRz4 (D). At least three independent experiments were conducted.

Figure 4. 10

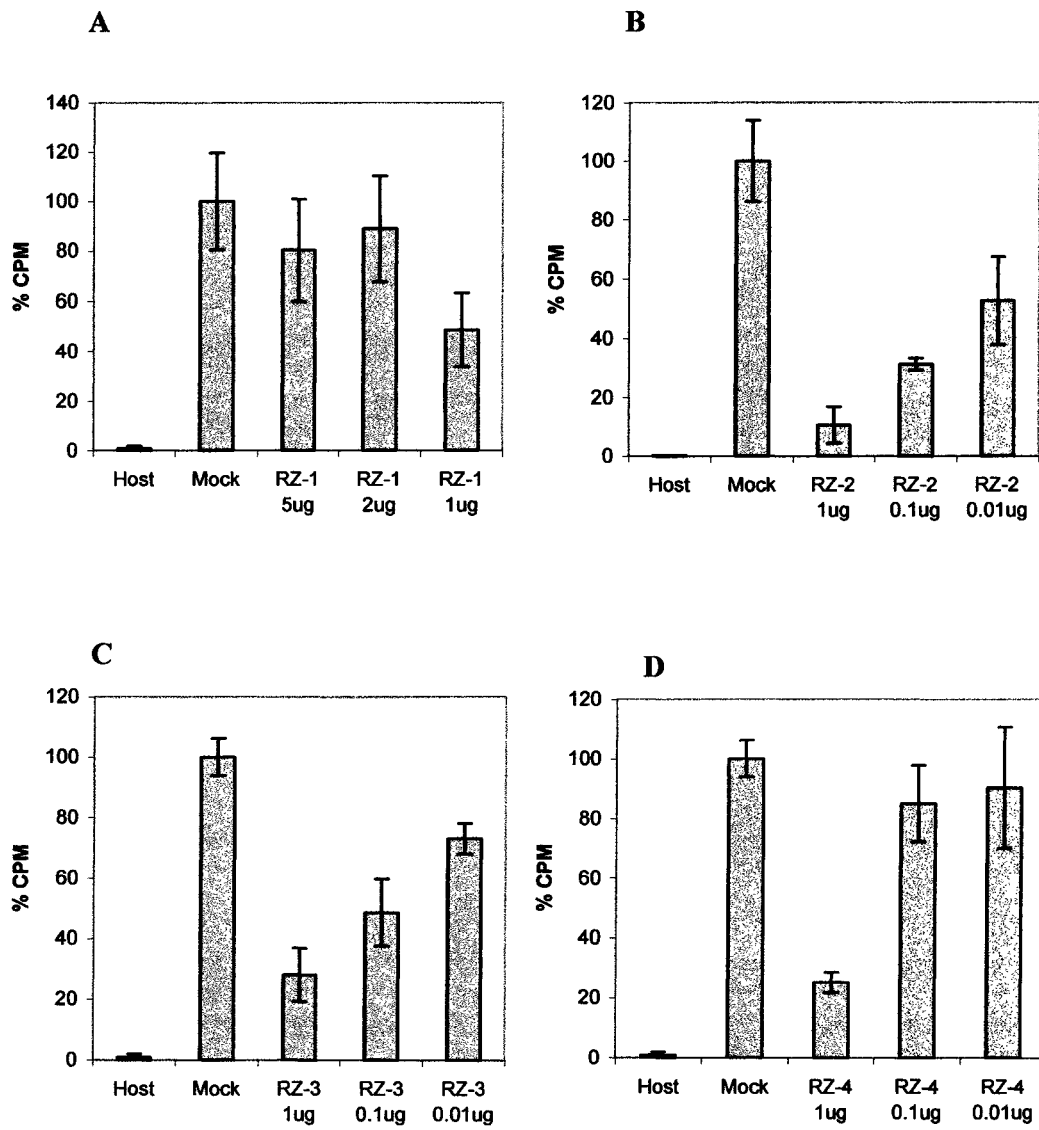


FIGURE 4.11

Effect of Supplemented WT *Trans*-acting Delta Ribozymes on UPRT activity

in *T. gondii*

Legend

Histogram illustrates the level of ^3H -uracil radioactivity in *T. gondii* electroporated with the engineered ribozymes 1 μg , 0.1 μg of engineered ribozymes mixed with 0.9 μg of WT *trans*-acting delta ribozymes, and 0.01 μg of engineered ribozymes mixed with 0.99 μg of WT *trans*-acting delta ribozymes. A. UPRz1, B. UPRz2, C. UPRz3, D. UPRz4. At least three independent experiments were conducted.

Figure 4. 11

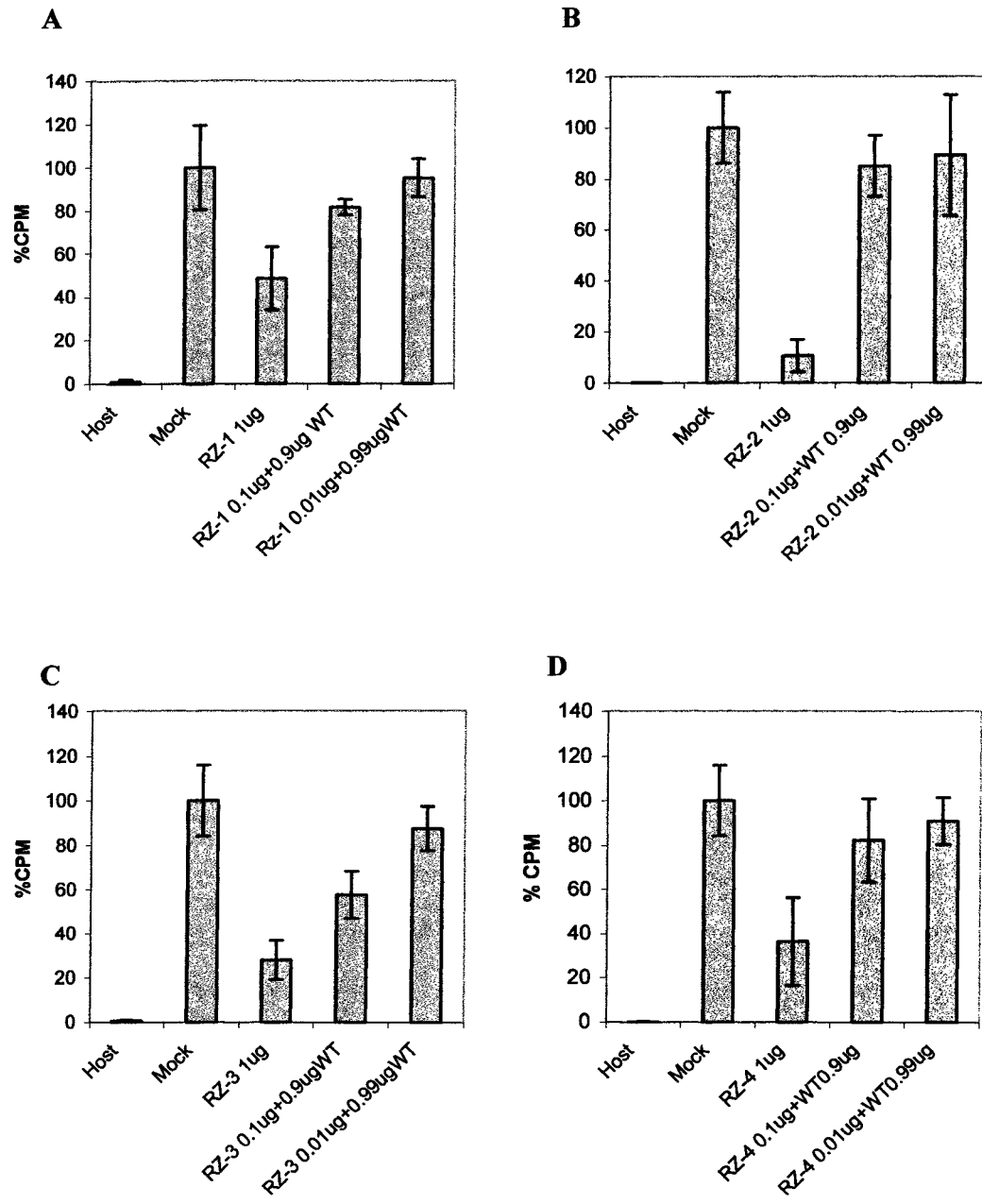


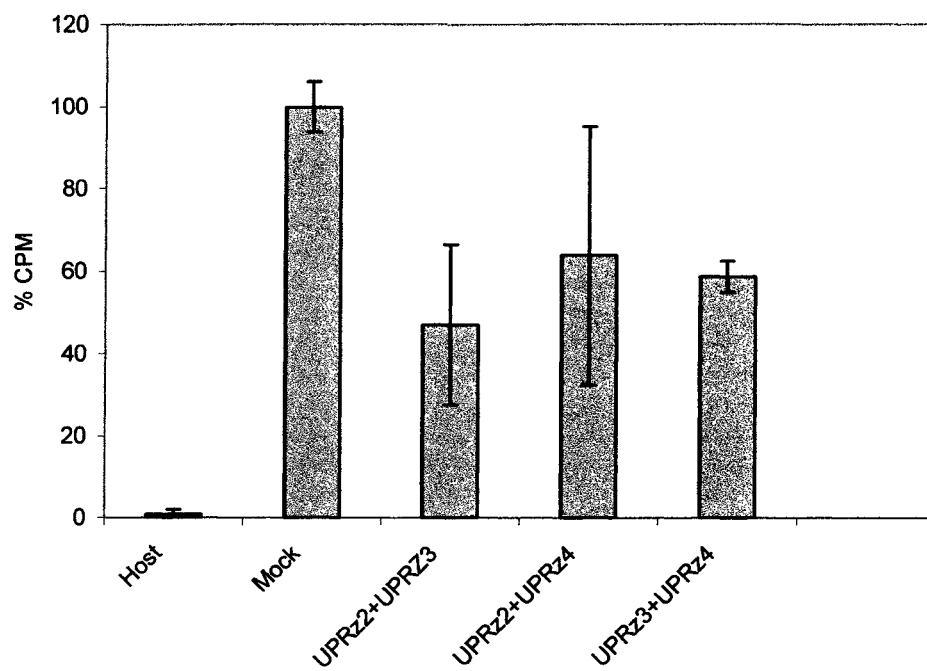
FIGURE 4.12

Effect of Mixture of Engineered Delta Ribozymes on UPRT Activity in *T. gondii*

Legend

Histogram illustrates the level of ^3H -uracil radioactivity in *T. gondii* electroporated with equal amount (0.5 μg) of UPRz2 with UPRz3, UPRz2 with UPRz4, and UPRz3 with UPRz4. At least three independent experiments were conducted.

Figure 4. 12



4.2.2 Effects of engineered ribozymes on the level of *HXGPRT* gene expression

HXGPRT activity of *T. gondii* was measured by hypoxanthine uptake experiment. As with the uracil incorporation assay, the relative HXGPRT activity was calculated as the ratio to the mock electroporation. Figure 4.13 showed that HXGPRT activities measured for parasites individually electroporated with equal amounts of HXRz1 and HXRz2 (1 µg) were lower compared with mock electroporation (30.7%, 11.1% respectively), while the same amount of control RNAs, yeast tRNA and wild type *trans*-acting delta ribozyme, did not show any effect on hypoxanthine uptake. One microgram of ribozymes specifically targetting *UPRT* mRNA (UPRz1, UPRz2, UPRz3, UPRz4) were also tested under the same conditions, but had no effect on hypoxanthine incorporation in *T. gondii*.

4.2.3 Antisense effect of deoxyoligonucleotides on *UPRT* and *HXGPRT* activity

In order to verify whether the antisense oligonucleotides could produce effects similar to those of the engineered ribozymes, deoxyoligonucleotides having complimentary sequences to the target sites on *UPRT* and *HXGPRT* mRNA were synthesized. Upon the electroporation of 53 pmols, which is molar equivalent to 1 µg of the ribozymes into parasites, *UPRT* and *HXGPRT* activities were measured by the ³H-uracil and ³H-hypoxanthine uptake assay respectively. No down regulation effect was observed with deoxyoligonucleotides for all sites tested (Fig. 4.14A). This clearly demonstrated that the decrease of *UPRT* and *HXGPRT* activity in the parasites electroporated with engineered delta ribozymes was not due to the antisense effect.

FIGURE 4.13

Effect of Engineered Ribozymes on HXGPRT Activity in *T. gondii*

Legend

Histogram illustrates the level of ^3H -hypoxanthine incorporation by *T. gondii* parasites electroporated with the engineered ribozymes. One microgram of HXRz1, HXRz2, yeast tRNA, wild type trans-acting delta ribozymes, UPRz1, UPRz2, UPRz3, and UPRz4 were individually electroporated into the parasites. The HXGPRT activity was inhibited by the engineered ribozymes both HXRz1 and HXRz2, while the control RNAs and UPRz1, UPRz2, UPRz3, UPRz4 did not have any significant effect. At least three independent experiments were conducted.

Figure 4. 13

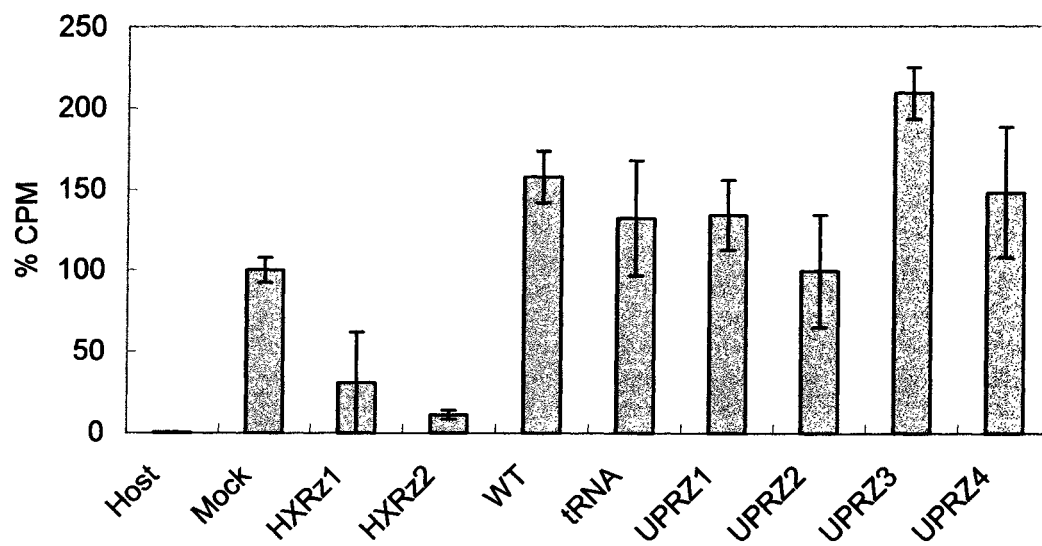


FIGURE 4.14

A. Antisense Effect of Deoxyoligonucleotides on UPRT and HXGPRT Enzymatic Activity

Legend

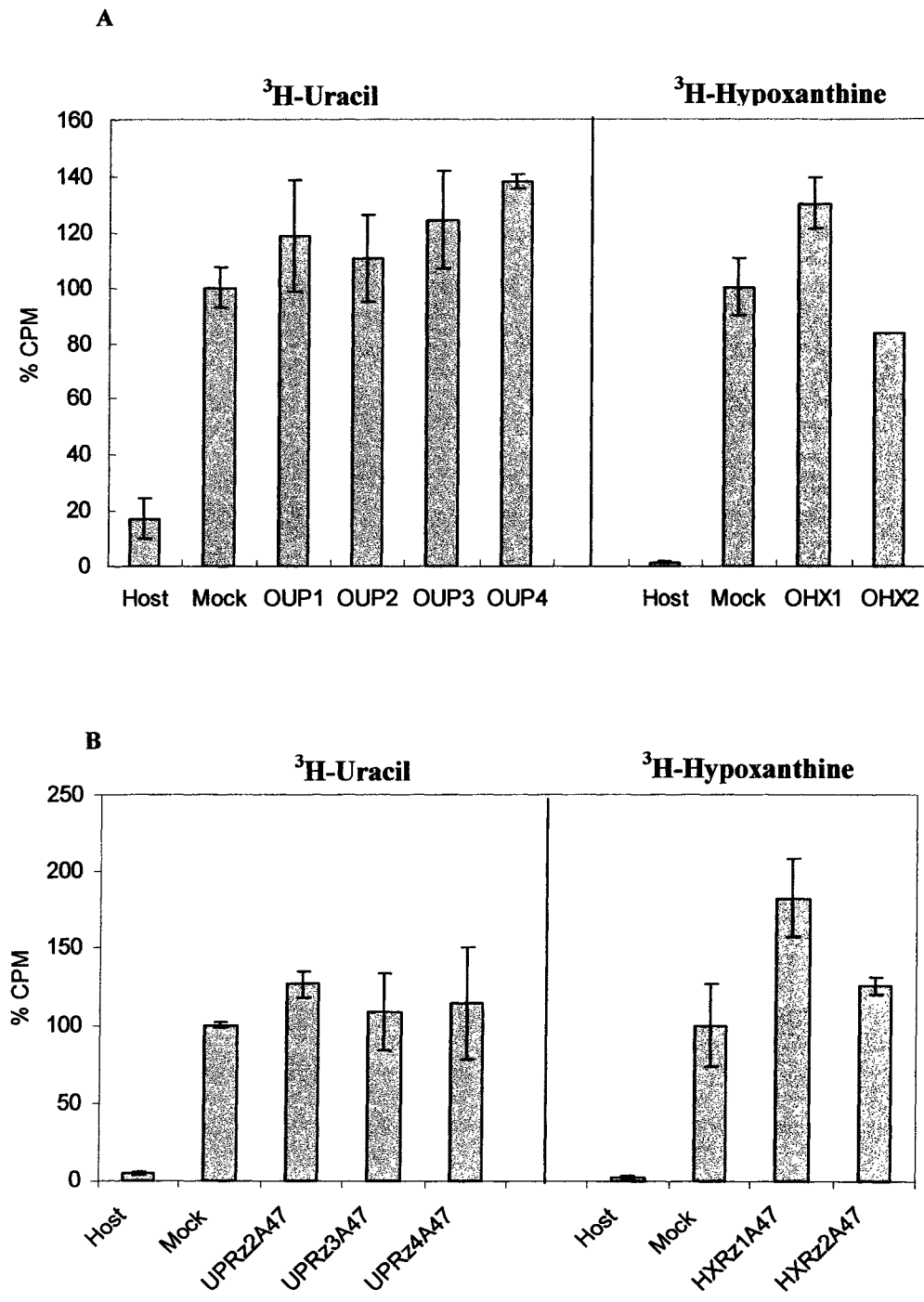
Histogram shows the level of ^3H -uracil and ^3H -hypoxanthine radioactivity in *T. gondii* electroporated with deoxyoligonucleotides having the complimentary sequences to the different selected target sites for UPRz1 UPRz1, UPRz2, UPRz3, UPRz4 on the target *UPRT* mRNA and the same locations as HXRz1, and HXRz2 on the target *HXGPRT* mRNA. At least three independent experiments were conducted.

B. Effect of Inactive Delta Ribozyme on UPRT and HXGPRT Activity

Legend

Histogram shows the level of ^3H -uracil and ^3H -hypoxanthine radioactivity in *T. gondii* electroporated with 1 μg of UPRz2-A47, UPRz3-A47, UPRz4-A47, HXRz1-A47, and HXRz2-A47. Uracil and hypoxanthine incorporation data showed that all the inactive ribozymes have no effect on UPRT and HXGPRT activity. At least three independent experiments were conducted.

Figure 4.14



4.2.4 Effect of inactive delta ribozyme on UPRT and HXGPRT activity

By changing one critical point base C47 to A47 in delta ribozyme, the active ribozyme becomes inactive even though it maintains the same substrate recognition sequence (Lafontaine et al., 1999). Equal amounts (1 µg) of inactive synthesized ribozymes UPRz2-A47, UPRz3-A47, UPRz4-A47, HXRz1-A47, and HXRz2-A47, were and electroporated into the parasites. Data from the uracil incorporation experiment showed that all the tested UPRz-A47 ribozymes had no effect on UPRT activity (Fig. 4.14B). Similar results were obtained from hypoxanthine incorporation assay using ribozymes, HXRz1-A47 and HXRz2-A47, which had no effect on HXGPRT activity.

4.2.5 Effect of engineered ribozymes on the expression of two genes in *T. gondii*

In order to investigate the knock-down effect of engineered delta ribozymes against *UPRT* and *HXGPRT* targets simultaneously, a mixture of equal amounts of active ribozyme UPRz2 and HXRz2 were electroporated into the freshly lysed parasites. UPRT and HXGPRT activities were measured. The results showed that uracil incorporation level was decreased by the mixture of UPRz2 and HXRz2 as compared to mock electroporation ($9.9 \pm 3.6\%$). This decrease was almost the same as decrease obtained with UPRz2 alone ($8.3 \pm 4.6\%$). On the other hand, HXRz2 had no effect on uracil assimilation just as expected (Fig. 4.15A). Similarly, hypoxanthine uptake level compared with mock was lowered by UPRz2 and HXRz2 mixture ($16.8 \pm 8.8\%$), the decrease had no significant difference compared with HXRz2 alone (11.0

$\pm 2.9\%$). This data demonstrated that UPRz2 and HXRz2 could function simultaneously in a specific and efficient manner.

To ensure that the down-regulation effects reported above were indeed the result of *UPRT* and *HXGPRT* mRNA cleavage and degradation by the delta ribozymes, RNAs extracted from the parasites electroporated with 1 μ g of UPRz2, HXRz2 and their mixture were subjected to Northern blot analysis. Mock electroporation was used as the control. As shown in Figure 4.15B, *UPRT* transcripts (2.2 kb) from parasites electroporated with both UPRz2 alone (Lane 2) and the mixture of UPRz2 with HXRz2 (Lane 4) were undetectable when hybridized with a DIG-labeled probe specific to *UPRT* cDNA, while the signals were detected in the samples of mock (Lane 2) and HXRz2 parasites (Lane 3). When the same blot was re-hybridized with *HXGPRT* specific probe, the *HXGPRT* transcripts (1.5 and 1.3 kb, corresponding to the two alternatively spliced transcripts of *HXGPRT*) from parasites electroporated with either HXRz2 alone (Lane 3) or the mixture of HXRz2 with UPRz2 (Lane 4) were also undetectable. Mock electroporation had no effect on *UPRT* and *HXGPRT* transcripts as evidenced by the signals detected for *UPRT* and *HXGPRT* with the sample electroporated with buffer.

To control for the quantity of RNA loaded per well, the blot was finally stripped and re-probed with ROP1, which is a housekeeping gene in *T. gondii*. The hybridization signal (2.1 kb) which was detected for each RNA sample confirmed that the lanes were loaded with comparable amounts of total RNA.

FIGURE 4.15

Effect of Engineered Ribozyme on the Expression of Two Genes (*UPRT* and *HXGPRT*) in *T. gondii*.

Legend

A. Histogram of both uracil incorporation and hypoxanthine uptake levels in parasites electroporated by a mixture of UPRz2 and HXRz2, and UPRz2, HXRz2 individually. At least three independent experiments were conducted.

B. Northern blot of RNAs extracted from the parasites electroporated with the mixture of UPRz2 and HXRz2

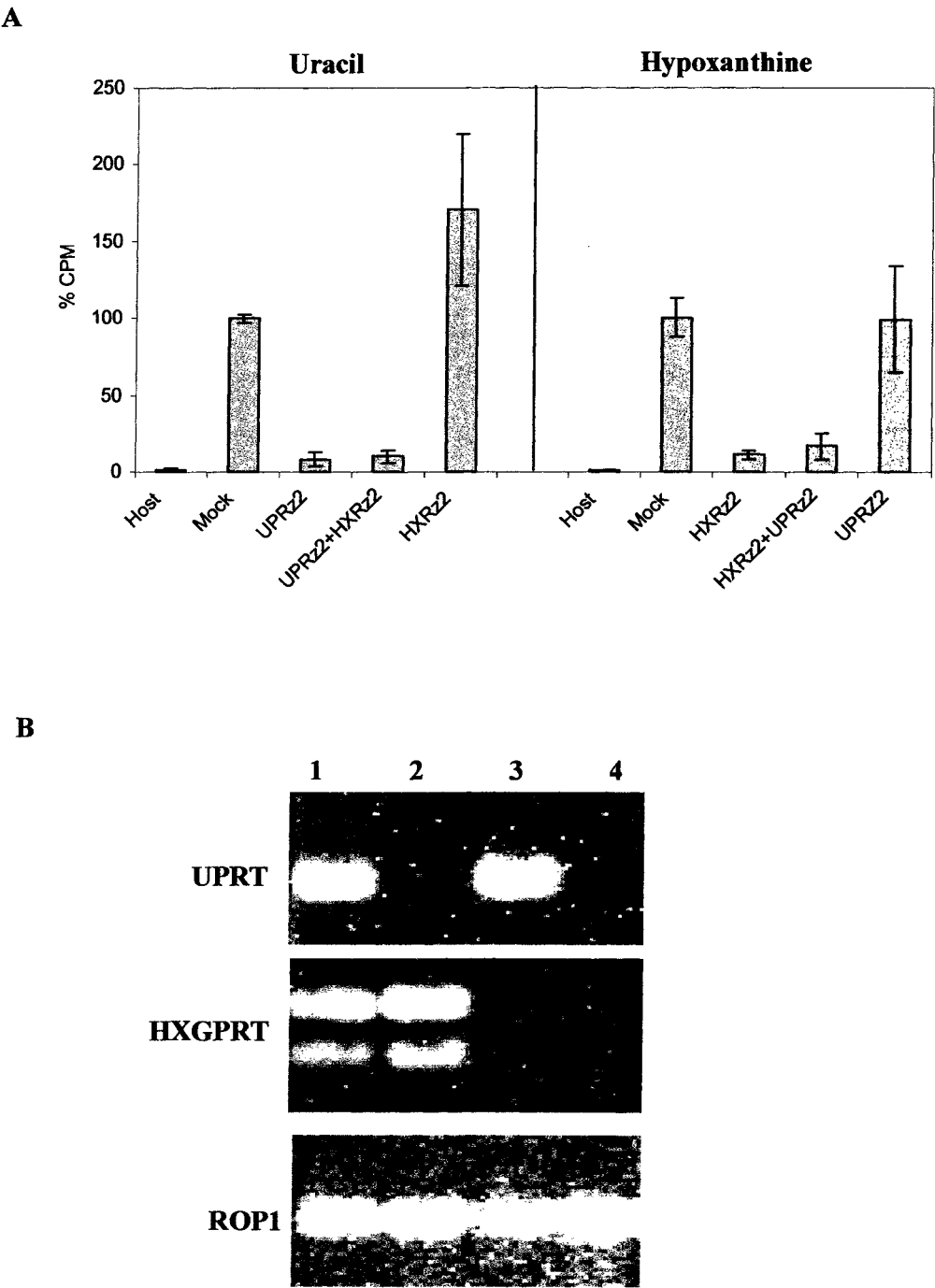
Lane 1 RNA extracted from parasites electroporated with buffer only (Mock electroporation)

Lane 2 RNA extracted from parasites electroporated with UPRz2

Lane 3 RNA extracted from parasites electroporated with HXRz2

Lane 4 RNA extracted from parasites electroporated with a mixture of UPRz2 and HXRz2

Figure 4.15



4.3 Characterization of a stable cell line of *T. gondii* expressing the engineered delta ribozymes against *UPRT*

The plasmids carrying the cDNA of engineered delta ribozymes with SAG1 promoter were electroporated into *T. gondii* RH strain. The stable transformed parasites were selected by passing the parasites in the completed medium with 5 μ M FDUR. To confirm the maintenance of plasmid in the transformed parasites, total DNA was extracted and then subjected to PCR using universal and reverse primers. Same amount of DNA sample (100 ng) extracted from RH parasites was used as a control. The PCR product was detected in the transformed parasites with unique band (750 nt) as expected to contain ribozyme sequence. The wild type sample did not give that unique band (Fig. 4.16A). Other two bands (200 nt, 1800 nt), showed in samples of both transformed parasites and RH parasites (Lane 2 and Lane 3), were non-specific PCR products. The data indicated that the constructed plasmids were maintained in the transformed *T. gondii* parasites. Southern blotting (using genomic DNA extracted from the transformed parasites) was carried out to further confirm the presence of the constructed plasmids. However, only the positive control, undigested pUC19-RZ2 (1 μ g), could be detected when hybridized to DIG-labeled probe specific to delta ribozyme. Extracted genomic DNA digested with *BamH* I did not give any signal (Figure not shown).

The expression of the ribozyme was detected by RT-PCR. Total RNA from the transformed parasites and wild type was extracted and quantified. Equal amount of each RNA sample (1 μ g) was subjected to reverse transcription for one hour at 37°C

to produce the corresponding cDNA. The RT products were then amplified by PCR and electrophoresed on 10% polyacrylamide gel. Plasmid pUC19-UPRZ2 was used as positive control in PCR. Figure 4.16B shows that 70 nt ribozyme fragment, same size as PCR amplicon of positive control, could be amplified from RNA of transformed parasites. No same size band was detected from control RH sample.

UPRT and HXGPRT activity of the stable cell line parasites (10^6 cells) were measured. Fig. 4.16C showed that uracil incorporation in these drug resistance parasites was dramatically lowered compared with RH parasites, while hypoxanthine incorporation were not dropped. Results of the above two experiments indicated engineered delta ribozymes were expressed in the stable cell line parasites.

FIGURE 4.16

Analysis of ribozyme expression in a stable cell line of *T. gondii*

Legend

A. Ethidium bromide stained agarose gel (1.0%) resolving PCR amplification products with reverse and forward primers spanning a 750 bp long region on plasmid pUC19-UPRz2

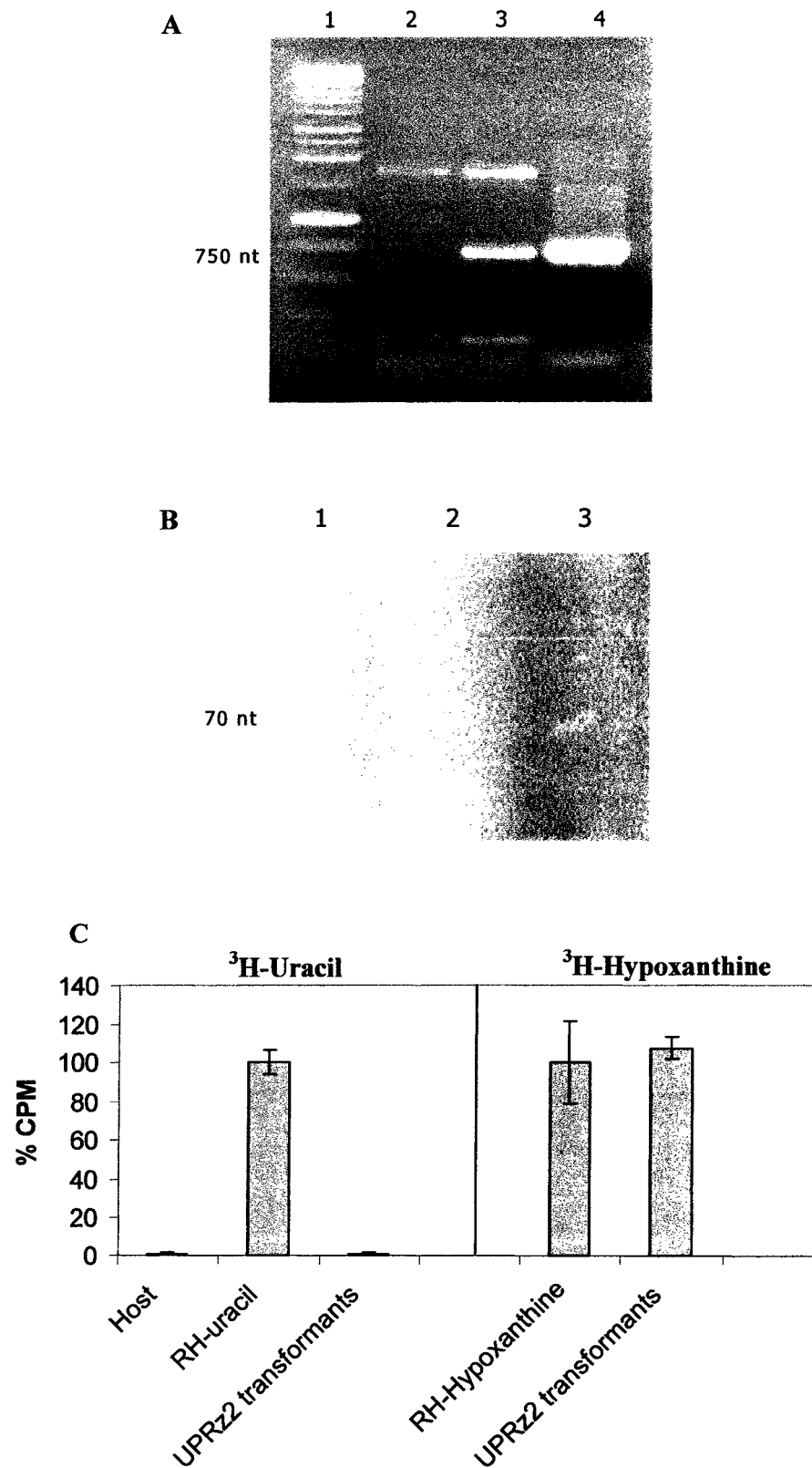
Lane 1 is 1 kb marker. Lane 2 is PCR product obtained using genomic DNA from wild type *T. gondii* as template (negative control). Lane 3 is PCR product obtained using genomic DNA from *T. gondii* parasites transformed with delta ribozyme. Lane 4 is PCR product obtained using plasmid pUC19-UPRz2 as template (positive control). The expected band was 750 bp long.

B. Ethidium bromide stained polyacrimide gel (10%) illustrating RT-PCR products with RT primer

Lane 1 is PCR product obtained using plasmid pUC19-UPRz2 as template (the amplified 70 nt long region included the ribozyme cDNA within this plasmid). Lane 2 is RT-PCR product using RNA from wild type *T. gondii* (negative control). Lane 3 is RT-PCR product using RNA from delta ribozyme *T. gondii* transformants.

C. Histogram of both uracil incorporation and hypoxanthine uptake levels in a stable cell line parasites compared with RH parasites. At least three independent experiments were conducted.

Figure 4.16



CHAPTER 5

DISCUSSION

5.1 Engineered delta ribozyme cleavage activity of *UPRT* mRNA *in vitro*

We engineered delta ribozymes to recognize and subsequently cleave *UPRT* mRNA. The initial step in the engineering of ribozymes was the selection of the target site with the greatest cleavage potential. Currently, three strategies are commonly used for choosing the potential target sites for delta ribozyme, these are (i) the use of bioinformatic tools, (ii) RNase H hydrolysis analyses within a library of DNA oligonucleotides, and (iii) cleavage assays using a library of ribozymes (Bergeron and Perreault, 2002). The combinational method was recommended to be the best means. However, the last two methods are quite labor and time consuming, they are not widely accepted in ribozyme designing. By using a computer program, named MFOLDTM, the most-probable RNA secondary structures can be predicted from the cDNA sequence of the target RNA and minimum free energy parameters. The accuracy of this thermodynamic modeling method is over 80% (Zhao et al., 1998). In this study, we chose four sites in *UPRT* cDNA according to the predicted secondary structure of *UPRT* mRNA. Ribozymes were synthesized to cleave these selected potential cleavage sites by changing their recognition sequence (P1 stem) into the complimentary sequence of the target mRNA.

The effective cleavage at the appropriate target primarily depends on whether the ribozyme can access the target site (Campbell et al., 1995; Birikh et al., 1997). Ananvoranich and Perreault (1998) found that factors such as tertiary structure

interactions could play an important role in the substrate specificity of delta ribozyme cleavage. It has been demonstrated that, for the model substrate, the nucleotide composition at positions -1 to -4 (upstream of cleavage site) influences the efficiency of cleavage (Ananvoranich et al., 1999). That might explain why some of the engineered ribozymes exhibited poor or even no cleavage of the target RNA. In the work of Bergeron et al. (2002), only two out of the nine ribozymes tested showed cleavage activity for *in vitro* delta ribozyme cleavage assay. Based on the predicted secondary structure, it might be possible to decide the better target site. For example, the single strand in the loop area is likely to be more accessible to ribozyme binding than the double-strand regions. However, one of the limitations for the target selection according to the predicted secondary structure is that it can not take into account the tertiary structure of both the ribozyme and the RNA target. In an attempt to test the engineered delta ribozyme activity, we first used model substrates (15 nt long) under single-turnover conditions. We detected that only one ribozyme, namely UPRz2, could cleave its model substrate (Fig. 4.3). The most active ribozyme (UPRz2) was then used in kinetic studies and the subsequent *in vivo* studies. UPRz1 was found unable to produce detectable product in primer extension reaction when the long *UPRT* substrates were used to test the cleavage activity of engineered ribozymes. It might be because of its location in *UPRT* mRNA. Other ribozymes showed less active than UPRz2 supported by both primer extension and uracil incorporation experiments.

It is intriguing that UPRz2 could function in the presence of cell-free extract. The observed rate of cleavage reaction with cell-free extract was $0.047 \pm 0.031 \text{ min}^{-1}$ with

the same concentration of ribozyme (over two times compared to that without cell-free extract). The improvement in the kinetic parameters of UPRz2 indicates that *T. gondii* proteins have some effects on the ribozyme activity and hence might aid in the binding and cleavage of UPRz2. The interaction of the cellular protein with delta ribozyme might be a significant factor that affects the stability and activity of delta ribozyme.

5.2 Transient knockdown of gene expression by engineered delta ribozyme in *T. gondii*

Another limitation for the ribozyme design based on the predicted secondary structure is that the software can not take into account the RNA-protein interaction, which is one of the most important factors in living cells. The additional intracellular factors, such as different pH also should be considered when comparing results from experiments conducted under *in vitro* versus *in vivo* conditions because the intracellular environment might influence ribozyme activity. It has been suggested that the rate-limiting step in ribozyme-mediated reactions *in vivo* is substrate-binding step (Castanotto et al., 2000; Warashina et al., 2001). Comparing ribozyme activities *in vitro* and in cultured cells, Koto et al. (2001) provided supporting evidence that the efficiencies of ribozymes *in vivo* depend more strongly on the expression system and the localization within cells than on cleavage activities *in vitro*. In their experiment, the tested delta ribozymes with very limited activities *in vitro* showed significant inhibitory effects in cultured cells. Similar result was reported from the study on

hammerhead ribozyme activity in the presence of low molecular weight cellular extract (Nedbal et al, 1997), which further suggests that some cellular factors may facilitate ribozyme activity *in vivo*. Uracil incorporation data indicated that UPRT activity was lowered by ribozymes that could not give detectable cleavage products during the *in vitro* experiments. Our results are thus consistent with the previous work and provide more experimental support as to the conclusion that cellular factors may facilitate ribozyme activity *in vivo*.

It was observed that when UPRz was electroporated into parasites, the hypoxanthine incorporation was increased. The electroporation might result in higher level of nucleobase in cell and it might subsequently activate the HXGPRT activity. Since *T. gondii* can *de novo* synthesize pyrimidine, there is no increasement on UPRT activity.

It has been reported that multiple hammerhead ribozymes can be expressed within the same RNA transcript (Ramezani et al., 1997). Roy et al. (1999) reported that *trans*-acting delta ribozyme was able to cleave HDAg mRNA efficiently at several target sites. Delta ribozyme has been used to target the HCV RNA and the cleavage effects could be improved by applying different *trans*-HDV ribozymes to cleave HCV RNA at multiple target sites simultaneously *in vitro* (Yu et al., 2002). To investigate whether two active engineered delta ribozyme could enhance the down regulation effects on *UPRT* target gene *in vivo*, we combined two of UPRz2, UPRz3, and UPRz4 to transfect the parasites. Results (Fig. 4.12) suggest that the engineered ribozymes could function at the same time, but somehow interfere with each other's

action. One possible reason is that some limited factors, such as Mg^{2+} , are needed by both ribozymes to function in the cell, while there are not as excess amount as *in vitro* environment.

The question that arises here is whether the down-regulation function of delta ribozymes is only an antisense effect. As reviewed in chapter 1, gene suppression of ribozyme is probably mediated by a combination of two steps: the first step involves interaction through Watson-Crick base pairing, which could be altered to suit any target RNA sequence and in a second step, ribozyme catalyze destruction of the mRNA target. To verify whether the down-regulation effect of engineered ribozymes is not simply caused by substrate-binding, electroporation with equal molar deoxyoligonucleotides complimentary to target *UPRT* and *HXGPRT* mRNA was performed separately. No significant down-regulation effect was observed for all target sites tested (Fig. 4.14A). This eliminates the possibility of antisense effect by the engineered delta ribozymes.

It was reported that active ribozyme becomes inactive by changing the cytosine (C) residue at position 47 of the trans-acting delta ribozymes to adenine (A) and the new ribozymes maintained the same substrate recognition sequence but lost the cleavage activity (Lafontaine et al., 1999). We synthesized inactive ribozymes with same sequence as UPRz2, UPRz3, UPRz4, HXRz1 and HXRz2 with an exception involving the change of one critical point base C47 to A47 in delta ribozyme. UPRT and HXGPRT activity was not down regulated in the parasites electroporated with the synthesized inactive ribozymes (Fig. 4.14B). It is unlikely that the inhibition of either

UPRT or *HXGPRT* expression resulted from the effect of antisense or only inactive structure. Therefore, we conclude that the reduction in ^3H -uracil or ^3H -Hypoxanthine incorporation is a consequence of a deficit in *UPRT* or *HXGPRT* function brought about by the cleavage activity of engineered delta ribozymes.

During the investigation of knock-down effect of ribozymes on two different targets simultaneously, we found that two active engineered ribozymes, UPRz2 and HXRz2, could lower the incorporation of uracil and hypoxanthine as much as when individual ribozymes were used (Fig. 4.15A). Northern blot results (Fig. 4.15B) ascertained that the reduction of *UPRT* and *HXGPRT* enzymatic activity is due to inhibited expression of *UPRT* and *HXGPRT* mRNA. It is demonstrated that UPRz2 did specifically indeed degrade *UPRT* mRNA and HXRz2 could only cleave *HXGPRT* mRNA. The data is consistent in support of the fact that the delta ribozymes can be engineered against two independent genes in *Toxoplasma*. This finding might be valuable for future studies which focus on the application of delta ribozymes to knock-down two or more oncogenes.

5.3 A stable cell line of *T. gondii* expressing the engineered delta ribozymes against *UPRT*

We obtained a stable cell line of *T. gondii* expressing the engineered delta ribozymes by screening them in the medium with FDUR. PCR result (Fig. 4.16A) showed that the plasmids carrying engineered delta ribozymes existed in the transformed parasites. However, the constructed plasmids were not detected by

Southern blot. One reason for this could be that a small amount of constructed plasmids maintained in the transformed parasites. The expression of delta ribozymes in these parasites could be detected by RT-PCR (Fig. 4.16B). Also the UPRT activity became very low in the stable cell line parasites (Fig. 4.16C). It demonstrated that the plasmids with cDNA of engineered delta ribozymes, even though only a little amount were maintained in the parasites, were expressed steadily under control of the modified promoter SAG1.

In conclusion, the engineered delta ribozymes displayed a cleavage activity *in vitro* and down regulation effect on the gene expression in *T. gondii*. The engineered delta ribozymes could also function on two different targeted genes simultaneously. Because it is effective and simple, future study should be on the application of delta ribozymes among apicomplexan parasites or on the other oncogene that related to human disease. Especially the finding that the engineered delta ribozymes could down regulate two genes (*UPRT* and *HXGPRT*) simultaneously suggest us the future use of the engineered delta ribozymes to down regulate two or more oncogenes.

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Appendix

Oligonucleotide Primers Used in the Study

Oligonucleotide Name	Sequence of the Oligonucleotide (5' to 3')
Primers for the construction of ribozymes	
UPRz1 Upper	GTCGGGGATGGGCATG
UPRz1 Lower	CCCATCCCC
UPRz2 Upper	GTCTTCGGTGGGCATG
UPRz2 Lower	CCCACCGAA
UPRz3 Upper	GTCTCTGGAGGGCATG
UPRz3 Lower	CCCTCCAGA
UPRz4 Upper	GTCGAGCCTGGGCATG
UPRz4 Lower	CCCAGGCTC
HXRz1 Upper	GTCGTAGTTGGGCATG
HXRz1 Lower	CCCAACTAC
HXRz2 Upper	GTCTGTGGAGGGCATG
HXRz2 Lower	CCCTCCACA
Primers for synthetic substrate	
PST T7 Oligo	GCCTGCAGTAATACGACTCACTATAG GG
ComSub_1_15_long	ATCGGGGATCGACCCTATAGTGAGTC GTATTAC
ComSub_2_15_long	TCCTTCGGTGTTCCCTATAGTGAGTC GTATTAC
ComSub_3_15_long	CTCTCTGGATGACCCTATAGTGAGTC GTATTAC
ComSub_4_15_long	GCCGAGCCTCAGCCCTATAGTGAGTC GTATTAC
Primers for <i>UPRT</i> PCR	
T7on5'UP	CATTGGATCCATGGCGCAGGTCCCAG
3'UPRT	C CCAAAGTACCGGTCACCG
Primers for <i>HXGPRT</i> PCR	
T7on5'HX	ATGGCGTCCAAACCCATTGAA
3'HXGPRT	ACCGGTGTCGACGTCCTC
Primers for Primer Extension Experiment	
UPRz-1 3'Product	TTCATGAGCACCACATTGGG
UPRz-2 3'Product	CACTTCCTTCTTTTCGAACGG
UPRz-3 3'Product	CACCCAGCGATCTCTAATGTC
UPRz-4 3'Product	CAGGCAGATGTCAACAGCAGCA

Oligonucleotides for Electroporation

Com_sub_RNaseH RZ-1	ATCGGGGATCGA
Com_sub_RNaseH RZ-2	TCCTTCGGTGTT
Com_sub_RNaseH RZ-3	CTCTCTGGATGA
Com_sub_RNaseH RZ-4	GCCGAGCCTCAG
CHXS1	GCCGTAGTCTTG
CHXS2	TTCTGTGGATGT

Primers for Ribozyme PCR & RT-PCR

HxoI-RZ-FW	AAACTCGAGGGTCCACCTCC
KpnI-RZ-RV	TTTGGTACCGGGTCCCTTAG
RT-Primer	TCCCGGGTCCCTTAG
Universal Primer	CAGGAAACAGCTATGAC
Reverse	GTAAAACGACGGCCAGT

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